THE UNIVERSITY OF ALBERTA

AN INVESTIGATION OF CONTROLS ON SOIL PHOSPHATASE ACTIVITY UNDER LABORATORY AND FIELD CONDITIONS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF SOIL SCIENCE

EDMONTON, ALBERTA

SPRING, 1983

MICRO

THE UNIVERSITY OF ALBERTA

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NAME OF AUTHOR DAVID W. BERGSTROM

TITLE OF THESIS AN INVESTIGATION OF CONTROLS ON SOIL PHOSPHATASE ACTIVITY UNDER LABORATORY AND FIELD CONDITIONS

DEGREE FOR WHICH THESIS WAS PRESENTED M.Sc.

YEAR THIS DEGREE GRANTED 1983

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "An Investigation of Controls on Soil Phosphatase Activity under Laboratory and Field Conditions," submitted by David W. Bergstrom in partial fulfilment of the requirements for the degree of Master of Science.

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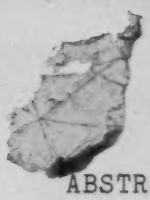

ABSTRACT

Controls on soil phosphatase activity were investigated in three studies, with the following objectives: (i) characterizing phosphatase activity in surface samples of two soils by determination of pH optima, and K_m and V_{max}; (ii) examining control of microbial phosphatase synthesis by orthophosphate in incubated soil samples; and (iii) examining temporal and spatial variation of phosphatase activity in soil, and relating controls on phosphatase activity at the microsite to the effect of crop, rotation, and fertilizer on enzyme levels in the field.

Phosphatase activity in samples of a Black Chernozemic soil and a Gray Luvisolic soil possessed an alkaline (pH 8-9) and a neutral (pH 6-8) pH optimum, respectively, indicating differences in the relative amounts of plant and microbial phosphatase present. Application of Michaelis-Menten kinetics yielded apparent K_m values of 2.2 and 2.3 mM, and apparent V_{max} values of 5.6 and 14.5 µmoles p-nitrophenol h^{-1} g^{-1} soil for the two soils. Discrepancies in fit of Michaelis-Menten kinetics to soil phosphatase activity measurements suggested a multiple enzyme-environment system.

Measurement of phosphatase activity, CO_2 evolution, and extractable P in incubated samples of a Gray Luvisolic soil which received two successive pulses of

nutrients (glucose and NH_4NO_3 with and without KH_2PO_4 in the first pulse, and treatments switched in the second pulse) indicated that added P repressed synthesis of acid phosphatase by the proliferating microbial population more than synthesis of alkaline phosphatase. In the treatment which received glucose, NH_4NO_3, and KH_2PO_4 in the first pulse followed by glucose and NH_4NO_3 in the second pulse, residual added P and stabilized phosphatase from the first incubation period prevented marked derepression of phosphatase synthesis in the second period.

In a Black Chernozemic soil near Ellerslie plant growth over the growing season increased levels of phosphatase in the rhizosphere. A single application of N at 80 kg ha^{-1} with and without P at 40 kg ha^{-1}, or P alone did not change soil phosphatase activity significantly. Acid phosphatase activity measurements on soil samples of the Breton Plots (representing 49 years of specific cropping systems and fertilizer applications) on a Gray Luvisolic soil indicated that the five-year rotation (wheat, oats, barley, hay, hay) produced higher levels of phosphatase than the wheat-fallow rotation. Fertilizer treatments which increased plant production and soil organic matter levels tended to increase soil phosphatase activity. Liming of the acid soil reduced acid phosphatase activity.

ACKNOWLEDGEMENT

I wish to thank my supervisor, Dr. W. B. McGill, for the guidance and assistance he provided over the course of this work. The financial support of the Alberta Agricultural Research Trust is gratefully acknowledged.

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INTRODUCTION

This thesis is a study of soil phosphatase activity. It consists of five chapters: the first chapter is a literature review, the next three chapters describe the experimental work and report the results obtained, and the fifth chapter is a brief summary of the conclusions of this study. Although each of the chapters describing the experimental work is an integral unit, discussion of results in chapters 3 and 4 incorporates findings of previous chapters. Furthermore, the literature review is supplemented by an introduction within each of chapters 2, 3, and 4.

The experimental work consisted of three studies: the first characterized phosphatase activity of surface samples of two soils in terms of pH optima and kinetic constants; the second investigated control of phosphatase activity by orthophosphate in incubated samples of soil in the laboratory (representing control at the microsite); the third examined expression of control of phosphatase activity by orthophosphate in the field, together with the effect of crop and fertilizer on soil phosphatase activity. The first two studies represented laboratory work, while the third study involved examination of soil samples from field plots, and tested some of the findings of the laboratory studies.

CHAPTER 1

LITERATURE REVIEW

1.1 The Nature of Soil Phosphatase Activity

1.1.1 Introduction

The name "phosphatase" is used to describe a broad group of enzymes which catalyze the hydrolysis of both esters and anhydrides of phosphoric acid. In soil the substrate molecules for these enzymes are phosphate esters. Three kinds of phosphatase have been shown to exist in soil (Eivazi and Tabatabai, 1977): phosphoric monoester hydrolase (EC 3.1.3), also called phosphomonoesterase, phosphoric diester hydrolase (EC 3.1.4), also called phosphodiesterase, and "phosphotriesterase," a type not classified by The Commission on Enzymes of the International Union of Biochemistry but shown to act upon the substrate tris-p-nitrophenyl phosphate. Most studies on soil phosphatase activity have been concerned with the two phosphomonoesterases—alkaline phosphatase (EC 3.1.3.1), and acid phosphatase (EC 3.1.3.2). Phosphodiesterase activity in soil has not been examined extensively; this enzyme catalyzes the initial hydrolysis of nucleic acids and phospholipids, compounds comprising much of the organic P fraction of microorganisms (Alexander, 1977) and plants (Bieleski, 1973).

Skujins (1976) stated that soil is a living system where all biological activities proceed through enzymatic processes. These processes may be intracellular or extracellular. The specific activity mediated by phosphatase is the mineralization of organic phosphates yielding orthophosphate (Figure 1). Phosphate esters primarily from animal remains and excretions, plant residues, and microbial biomass are hydrolyzed, and the resulting orthophosphate is absorbed by microorganisms, or remains in the soil solution where it may be quickly adsorbed or precipitated.

1.1.2 Assay methods

This section will briefly describe the methods for measurement of phosphatase activity in soils. The presence of the enzyme in soil is detected by the occurrence of the reaction catalyzed by it, and the amount of enzyme is estimated by the reaction rate (Galstian, 1974). In such assays the enzyme is not extracted from the soil matrix prior to measurement of activity. In addition, qualitative methods have been developed to test for phosphatase production by microorganisms isolated from soil (Krasil'nikov and Kotelev, 1957; Greaves et al., 1963; Greaves and Webley, 1965). There are few reports of activity measurements on phosphatases extracted from soil (Hayano, 1977; Batistic et al., 1980; Nannipieri et al., 1980).

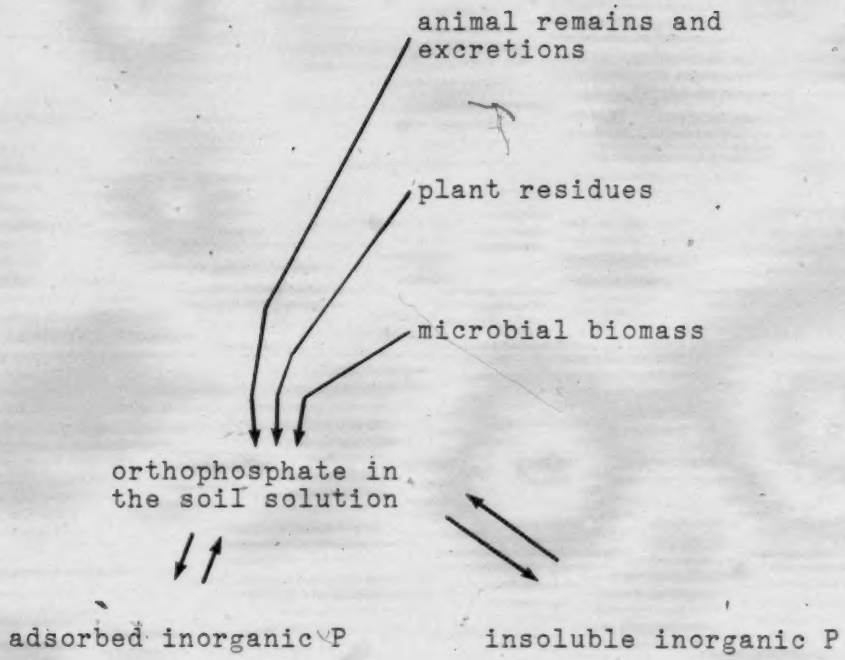


Figure 1. Some transformations of soil P, indicating the sources of substrate mineralized by phosphatase, and the fate of orthophosphate released.

The early studies of soil phosphatase activity employed natural substrates such as glycerophosphate, nucleic acids, and inositol phosphate, with activity measured by determining the inorganic phosphate released by hydrolysis. These substrates often required long incubation times, and hence use of a bacteriostatic agent (or sterilant) to prevent microbial proliferation and consequent assimilation of substrate and product during the assay. Toluene and ionizing radiation have been used to eliminate microbial proliferation during the assay. Methods depending on measurement of inorganic phosphate released by the reaction are complicated by adsorption of inorganic phosphate to soil components in the assay mixture. Drobnikova (1961) showed that sorption of inorganic phosphate by soils may occur in 2 or 3 stages. An artificial substrate—defined by Speir and Ross (1978) as a substrate neither identified as, nor suspected of being, a compound of soil organic P—was first used by Kroll et al. (1955). Artificial substrates have two advantages over natural substrates: the relatively low molecular weight esters used undergo more rapid hydrolysis, and the organic moiety released in the reaction is easily determined (Speir and Ross, 1978). Buffers are also used in assays of soil phosphatase activity to facilitate determination of activity at the pH optimum of the enzyme, and to prevent changes in pH during the assay which may affect reaction rate. The values obtained with artificial

substrates in buffered assay mixtures at higher temperatures are indices of potential phosphatase activity, and do not represent activity towards natural substrates *in situ*.

Kroll *et al*. (1955) developed a method for measurement of soil phosphatase activity using phenyl phosphate as substrate, in which 100-200 mg of phenyl phosphate dissolved in distilled water is added to 10 g of toluene-treated air-dry soil. After 24 hours the amount of liberated phenol is determined by the Folin-Ciocalteu method. Although the authors suggested that determination of orthophosphate remaining in the filtrate of the assay mixture provides an estimate of the biologically significant phosphatase activity, some of the orthophosphate released by enzyme activity is adsorbed by soil, and hence their hypothesis is tenuous at best. Kramer and Yerdei (1959) also used phenyl phosphate as a substrate. A 5 g sample of soil is shaken with 2.5 ml of toluene for 15 minutes, 20 ml of 0.5% disodium phenyl phosphate (pH 7) are added, and the mixture is incubated for 2 hours at 37 C. At the end of the incubation period 100 ml of 0.3% potash alum are added to the reaction mixture, which is subsequently filtered. For determination of phenol in the filtrate, an aliquot (0.5-2 ml) is treated with 5 ml of borate buffer (pH 9.4) and 4 drops of Gibbs reagent (2,6-dibromoquinone-chloroimide). The intensity of blue color formed is measured colorimetrically at 660 nm after 30 minutes.

Ramirez-Martinez and McLaren (1966a) developed a fluorimetric technique for determination of soil phosphatase activity in which a fluorogenic substrate (sodium β-naphthyl phosphate) is hydrolyzed by the soil enzyme yielding a fluorescent compound (β-naphthol) which can be measured directly in the soil extract. They showed that detection of β-naphthol is relatively unaffected by unhydrolyzed substrate and soil extract. Retention of product (β-naphthol) by soil must be taken into account for quantitative work. Furthermore, in some soils addition of NaOH to stop the reaction at the end of the incubation period and to raise the pH above 11 produces a dark colored extract in which β-naphthol fluorescence is masked by background fluorescence. This problem requires a modification of the method.

The method used in the present study is that of Tabatabai and Bremner (1969). It involves colorimetric determination of p-nitrophenol released by soil phosphatase activity when soil is incubated with buffered sodium p-nitrophenyl phosphate solution and toluene at 37 C for 1 hour. Tabatabai and Bremner (1969) compared their method using p-nitrophenyl phosphate as substrate with that of Kramer and Yerdei (1959) which uses phenyl phosphate. Results for samples of surface horizons of eight soils were similar, but usually larger values were obtained with p-nitrophenyl phosphate as substrate. It was emphasized that while the color developed from p-

nitrophenol was stable for 24 hours, the color developed during phenol determination using the method of Kramer and Yerdei (1959) was not stable for 24 hours, and strict control of temperature and pH was required. Cervelli et al. (1973) stated that in the method of Tabatabai and Bremner (1969) adsorption of substrate affects the activity measurement, and determination of Michaelis-Menten constant for soil phosphatase. They assumed that the reaction takes place in solution, and derived an equation for K_m determination which accounts for substrate adsorption.

Gerritse and van Dijk (1978) developed a method for determination of phosphatase activity in the presence of large amounts of organic matter such as occurs in surface horizons of soils and animal wastes. They use p-nitrophenyl phosphate as substrate, and the product formed (p-nitrophenol) is separated from the reaction mixture by high pressure liquid chromatography on a cellulose column to facilitate determination by absorptiometry without interference from dissolved organic matter or substrate. The method enables measurement of p-nitrophenol concentrations down to 0.1 uM, so that very low concentrations of substrate can be used. Further, because the authors showed that acid and alkaline phosphatase are strongly inhibited by orthophosphate at concentrations above 0.1 mM, substrate concentrations in the range of 0.01-0.1 mM were used. The measurements

can be corrected for adsorption of product (p-nitrophenol) by soil in the reaction mixture; adsorption isotherms should be determined for each soil used. The authors suggested that the phosphatase reactions may take place at particle surfaces due to adsorption of enzymes and substrate, and so omitted correction for adsorption of substrate.

There are few reports of determination of phosphodiesterase and phosphotriesterase activities in soils. Eivazi and Tabatabai (1977) and Hayano (1977) used bis-p-nitrophenyl phosphate as substrate in measuring phosphodiesterase activity of soil and soil extract respectively, determining the p-nitrophenol released in the reaction. Hayano (1977) showed that the extracted enzyme also hydrolyzed two nucleotide phosphodiesters—2'-deoxythymidine-3'-p-nitrophenyl phosphate, and 2'-deoxythymidine-5'-p-nitrophenyl phosphate. Browman and Tabatabai (1978) developed an improved method for assay of phosphodiesterase activity in soil which uses bis-p-nitrophenyl phosphate as substrate, and involves extraction of p-nitrophenol with 0.5 M $CaCl_2$ and 0.1 M tris-(hydroxymethyl)aminomethane (pH 12). These reagents give quantitative recovery of p-nitrophenol added to soil, and do not cause chemical hydrolysis of the substrate. In measurements of phosphotriesterase activity in soil, methylparathion (O,O-

dimethyl O-p-nitrophenyl phosphorothioate) (Kishk et al., 1976) and tris-p-nitrophenyl phosphate (Eivazi and Tabatabai, 1977) have been used as substrate.

A disadvantage of the use of p-nitrophenyl phosphate as substrate in determination of soil phosphatase activity has been described by Cosgrove (1977). He suggested that p-nitrophenyl phosphatase activity may not be correlated with phosphatase activity towards compounds such as inositol phosphates and nucleotides, which are alcoholic (as opposed to phenolic) esters. This problem may be overcome by the assay scheme of Rammler and Parkinson (1973) and Rammler et al. (1973) in which substrates such as the phosphate esters of 4-(p-nitrophenoxy)-1,2-butanediol and related diols are used. The diol released by hydrolysis of the ester is oxidized with periodate, yielding an aldehyde which is treated with a base to produce the p-nitrophenolate ion.

Harrison (1979) suggested that in studies where soils vary in bulk-density, it is more appropriate to express soil data (including phosphatase activity measurements) in terms of soil volume (cm^{-3} soil) rather than soil weight (g^{-1} soil). Furthermore, Harrison and Pearce (1979) stated that it was necessary to adjust phosphatase activity data to field temperature if the biological significance of phosphatase in the seasonal process of generating available P in soil is

to be appreciated. It may be argued that because phosphatase activity measurements indicate amounts of active enzyme present, and hence potential activities, adjustment of data to field temperature by itself will not provide estimates of biologically significant enzyme activity. Other factors such as substrate concentration and the nature of the soil microenvironment affect phosphatase activity *in situ*, and hence must also be considered.

Variation in methods for measurement of soil phosphatase activity has been the source of some disagreement in the literature concerning the nature of phosphatase activity in soil. Furthermore, Ramirez-Martinez and McLaren (1966b) suggested that contradictions in the literature may be due to an inadequate understanding of the limitations in the methods used, and a tendency to generalize too widely from experimental results. Berman (1969) pointed out the difference between *p*-nitrophenyl phosphatase activity (or that obtained with any artificial substrate) and the ecologically significant phosphatase activity. In samples of lake water the rate of release of orthophosphate from natural substrates was only 2.6-8% of the rate of hydrolysis of *p*-nitrophenyl phosphate. In spite of such limitations, the use of artificial substrates has facilitated determination of the controls on soil phosphatase activity.

Phenyl phosphate has become the most widely used artificial substrate (Speir and Ross, 1978).

1.1.3 pH optima of soil phosphatases

Determination of the optimum pH for phosphatase activity of soil samples provides indirect evidence about the source of enzyme in the samples. In a review discussing soil enzymes Skujins (1967) concluded that in spite of contradictory observations reported in the literature, in most cases maximum activity occurs at a nearly neutral value, and not necessarily at the natural pH of the soil. For example, Rogers (1942) found the optimum pH for dephosphorylation of nucleic acid by soil catalysts to be in the range 6.2-7.0. Kroll et al.(1955) found activity in manures to be highest in the range pH 5-8, and Drobnikova (1961) found that 3 of 4 soils tested showed optimum activity at a neutral or mildly alkaline pH. Similarly Halstead (1964), Ramirez-Martinez and McLaren (1966b), and Eivazi and Tabatabai (1977) have reported that most soils show optimum phosphatase activity around neutral pH. In a more recent review Speir and Ross (1978) stated that for soils where acid or alkaline phosphatase is present (or where both are present), the pH optima are generally within the ranges 4-6 and 8-10 respectively. In other soils there may be a "neutral" phosphatase with a broad peak about an optimum of pH 7. They suggested

that the latter may represent a mixture of acid and alkaline phosphatase. Several workers have found either acid or alkaline phosphatase to predominate. Halstead (1964) found peaks of optimum activity occurring at pH 5.0 and 9.5 in an organic soil, while Eivazi and Tabatabai (1977) reported that acid and alkaline phosphatase showed pH optima of 6.5 and 10 respectively, in the soils they examined.

Conflicting evidence has been presented about the relationship between the natural soil pH and the optimum pH for phosphatase activity. Kroll et al. (1955) found activity to be highest at the original soil pH, while Drobnikova (1961) reported that in only 2 of 4 soils studied did the optimum pH correspond to the natural pH. Eivazi and Tabatabai (1977) reported that acid phosphatase (pH 6.5) is predominant in acid soils while alkaline phosphatase (pH 10) is predominant in alkaline soils. Speir and Ross (1978) concluded that in most cases the optimum pH for phosphatase activity differs from the natural pH of the soil.

The variability in values reported for pH optima reflects differences in: (i) methodology, (ii) soils, and (iii) the source and microenvironment of enzyme within a single soil. Different pH optima are obtained with different buffers (Skujins, 1967; Speir and Ross, 1978). Speir and Ross (1978) pointed out that use of

artificial substrates tends to result in two pH optima while phosphatase activity measured with natural substrates appears to have only one pH optimum.

Determination of the optimum pH is also affected by the nature of the soil sample used. As Paulson and Kurtz (1970) showed for soil urease, the measured activity for a soil sample represents a microbial and adsorbed component; hence the relationship between soil phosphatase activity and pH would be expected to change with fluctuations in the microbial population as they affect the proportions of microbial and adsorbed enzyme present. Furthermore, pH optima for extracted phosphatase activity appear similar to those reported for soil samples; Batistic et al. (1980) found that extracted and purified acid and alkaline phosphatase (existing in soil partly as a carbohydrate-enzyme complex and partly as a humocarbohydrate-enzyme complex) had pH optima of 6 and 9 respectively.

The pH optima of root phosphatases of various plant species have been reported. Rogers et al. (1942) found that glycerophosphatase activity of corn roots was maximal at pH 4.0, while the pH optimum for dephosphorylation of nucleic acid by corn roots was 6.3. Estermann and McLaren (1961) found that sterile six-day barley roots showed highest glycerophosphatase

activity at pH 5.3. Woolhouse (1969) determined that the surface phosphatase activity of excised root tips of Agrostis tenuis was highest over the range pH 3.8-4.6, and Ridge and Rovira (1971) reported that the surface phosphatase activity of intact wheat roots (Triticum aestivum) was maximal at pH 4.5. Such evidence has led to the conclusion that in the soil-plant system roots are a source of acid phosphatase (Gould et al., 1979).

There are fewer reports in the literature of the pH optima of soil phosphodiesterase and phosphotriesterase. Kishk et al. (1976) reported that soil phosphotriesterase activity towards methylparathion was maximal at pH 7. Hayano (1977) extracted phosphodiesterase activity from samples of the A horizon of a forest soil, and found the activity to be highest in the range pH 5.2-6.0. Browman and Tabatabai (1978) showed that phosphodiesterase activity in several soil samples was maximal at pH 8.0.

Several conclusions can be drawn from the data cited above. The range in values suggests that there are different sources of phosphatase in soil. Furthermore, it is necessary in experimental work to determine the pH optimum of the phosphatase activity of the specific soil being studied, as opposed to selecting values from the literature. The data also suggest that in soil phosphatases may be active at a pH which is not optimal.

1.1.4 Sources of soil phosphatases

There are three general sources of phosphatases in soil (Skujins, 1967; Galstian, 1974; Ladd, 1978): proliferating and dying microorganisms, soil animals, and plant roots and residues. Although Speir and Ross (1978) suggested that microorganisms are the major producers of phosphatases in soil, the contribution of plants (particularly in the rhizosphere) cannot be discounted. Various organisms produce phosphatases in soil: bacteria, actinomycetes, protozoa, fungi, mycorrhizae, and plants. Kobus (1961) estimated that 5-80% of the total number of bacteria and actinomycetes in 12 different soils were capable of mineralizing glycerophosphate. Kotelev et al. (1962) investigated the hydrolytic activity of 32 species of bacteria, 35 species of fungi, and 8 species of actinomycetes towards nucleic acid or phytin in an agar medium at pH 7.0. Most bacterial and fungal species formed nuclease but not phytase, while actinomycetes did not show nuclease activity. Greaves et al. (1963) showed that 30-50% of microorganisms in soil and on plant roots possessed an enzyme capable of hydrolyzing sodium phytate under acidic conditions (pH 4.5-5.5). Genera possessing such activity included Aerobacter, Rhodotorula, Streptomyces, and Bacillus. Mazilkin and Kuznetsova (1964) classified various bacteria isolated from a gray forest soil into four groups

based upon their capacity to produce specific phosphatases (Table 1). Ko and Hora (1970) found phospholipase activity towards lecithin to be widespread among actinomycetes; more than 60% of the actinomycetes tested (isolated from 5 soils) produced phospholipase.

Protozoa are another source of phosphatase in soil. Gould et al. (1979), in a study of rhizosphere phosphatase activity, reported that cell-free extract of amoebae (Acanthamoeba sp.) contained acid phosphatase. The significance of phosphatase production by protozoa in soil is still being investigated. Using soil microcosms, Cole et al. (1978) showed that amoebal grazing resulted in a highly significant release of bacterial P. Although they hypothesized that the regeneration of bacterial P resulted from direct excretion of inorganic P by grazers, there was no direct measurement of amoebal phosphatase activity in their study.

Fungi also produce phosphatase, and may be significant sources of phosphatase activity in the organic layer of forest soils and in acid soils. Casida (1959) showed that Aspergillus niger possessed strong acid phosphatase activity with a pH optimum of 2.5 for hydrolysis of phytin, and 4.0 for hydrolysis of deoxyribonucleic acid. He suggested that these enzymes are probably active wherever growth of the fungus occurs. Greenwood and Lewis (1977) reported that 3 of 5 species

Table 1. Classification of some soil bacteria isolated from a gray forest soil according to production of specific phosphatases (from Mazilkin and Kuznetsova, 1964).

Bacteria	Phosphatases*
Bacillus adhaerens	high phosphatase
Pseudomonas spp.	activity
Bacteroides glutinosus	low nuclease and
Bacillus cereus var. *mycoides*	phosphatase activity
Mycobacterium spp.	
Micrococcus spp.	
Bacillus cereus	high ribonuclease and
B. megatherium	phosphatase activity
B. mesentericus	
Bacillus cereus	deoxyribonuclease and
B. subtilis	phosphatase activity
B. mesentericus	

* in their scheme, phosphatase activity refers to activity towards a substrate other than nucleic acid.

of soil yeast of the genus Cryptococcus grew on sodium-inositol hexaphosphate as the sole source of P. These three species hydrolyzed p-nitrophenyl phosphate, β-glycerophosphate, pyrophosphate, and sodium-inositol hexaphosphate, but not insoluble (ferric and aluminum) salts of inositol hexaphosphate.

Mycorrhizae are another source of phosphatases in soil. Bartlett and Lewis (1973) found that mycorrhizal roots of beech (Fagus sylvatica) produced surface acid phosphatases which hydrolyzed p-nitrophenyl phosphate, glucose-6-phosphate, β-glycerophosphate, inositol hexaphosphate, inositol triphosphate, and inorganic pyrophosphate. The mycorrhizal roots of Sitka spruce (Picea sitchensis) have also been shown to possess acid phosphatase activity (Alexander and Hardy, 1981).

Experiments demonstrating plant utilization of organic P compounds and hydrolysis of specific organic P substrates by plant roots in solution culture under sterile conditions provide evidence for plant root production of acid phosphatase. Rogers et al. (1942) found that enzymatic activity of corn roots was associated with the gelatinous material coating the roots in solution cultures, and concluded that phosphatase enzymes are held in the sloughed-off cellular material.

Lecithin and phytin can serve as the sole source of P for radish plants grown under sterile conditions (Szember, 1960). Estermann and McLaren (1961) showed that sterile six-day barley roots were efficient mineralizers of glycerophosphate, and concluded that the phosphatase activity of the root zone can be attributed in large part to the enzymes of the root itself. Saxena (1964) found that under sterile conditions roots of pea, gram, wheat, and barley possessed substantial phytase activity. Woolhouse (1969) showed that excised root tips of Agrostis tenuis possessed surface acid phosphatase activity which hydrolyzed p-nitrophenyl phosphate, ß-glycerophosphate, and inositol hexaphosphate. Ridge and Rovira (1971) reported that most of the phosphatase activity (towards p-nitrophenyl phosphate) of intact wheat seedling roots was associated with the root surface, and not increased by microorganisms in the rhizosphere. Roots of Bouteloua gracilis also produce acid phosphatase (Gould et al., 1979).

The information cited above indicates that phosphatase production is widespread among soil microorganisms, and has been demonstrated for a variety of plant species. Furthermore, the diversity of source of phosphatase in soil contributes to the nonspecific character of the aggregate phosphatase activity of a soil sample.

1.1.5 The state of phosphatases in soil

Skujins (1976 and 1978) and Kiss et al. (1975) have described various components of soil enzyme activity (including phosphatase activity) based upon the physical location of the enzyme. Figure 2, adapted from Skujins (1978), relates the various components. In this scheme the total activity includes that of intracellular enzymes, together with contributions from extracellular or "abiontic" enzymes (enzymes accumulated outside of proliferating cells and extracellular enzymes which are continuously released from cells in soil). Skujins (1967) emphasized that superimposed upon the variation in physical location of soil enzymes is spatial variation in microenvironment throughout the soil matrix, and in molecular environment at the surfaces of soil particles.

Evidence about the nature of soil phosphatases has been obtained by several experimental approaches: studies on adsorption of enzymes by mineral and organic components, extraction of enzymes from soil, and studies of kinetic parameters of soil enzymes (Speir and Ross, 1978). The kinetics of soil phosphatase activity will be discussed in chapter 2.

Adsorption of phosphatase has been shown to affect activity of the enzyme. Mortland and Gieseking (1952) investigated the influence of clay minerals on dephosphorylation of organic P compounds. All four clay

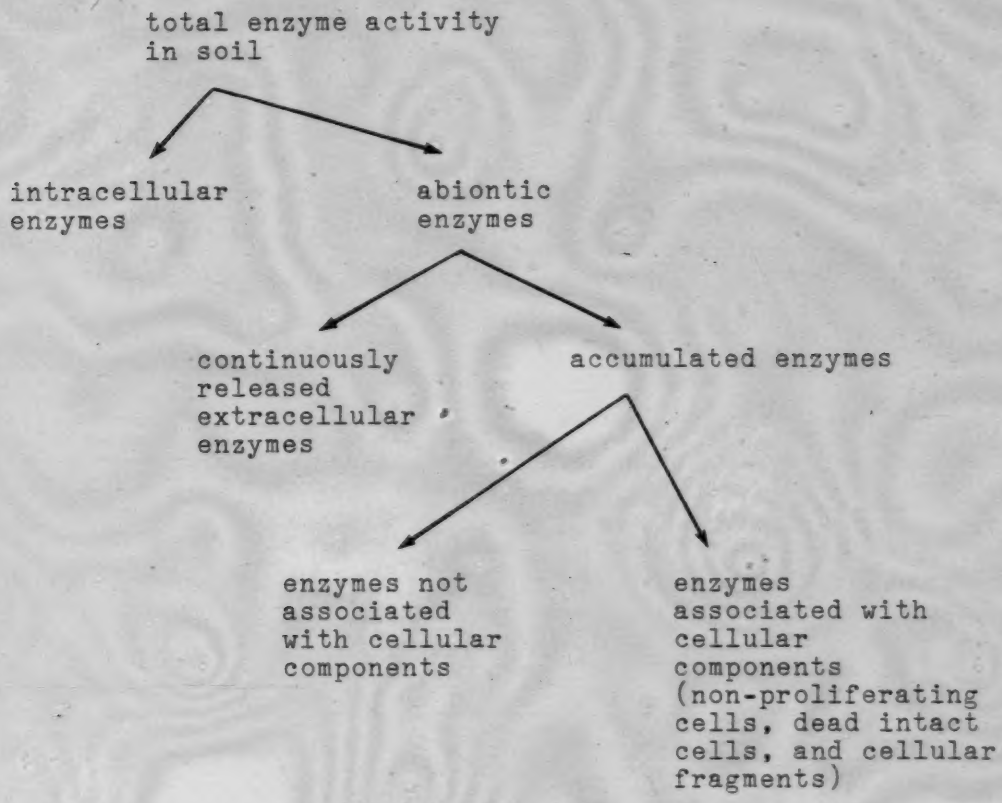

total enzyme activity in soil
intracellular enzymes
abiontic enzymes
continuously released extracellular enzymes
accumulated enzymes
enzymes not associated with cellular components
enzymes associated with cellular components (non-proliferating cells, dead intact cells, and cellular fragments)

Figure 2. Various components of soil enzyme activity, based upon the physical location of enzymes in soil and association with microorganisms, plant roots, and cellular debris (adapted from Skujins, 1978).

minerals tested (montmorillonite, a Putnam-like clay, illite, and kaolinite) inhibited the hydrolysis of the organic P compounds (fructose-diphosphate, phytin, glycerophosphate, and lecithin) by enzymes from bread yeast, wheat bran, and kidney cortex. The authors obtained evidence suggesting that the inhibition was due to the effect of the clay on the enzymes rather than the substrates. Moreover, the amount of inhibition was proportional to the base exchange capacity of the clay, suggesting that phosphatases may be adsorbed at least partially as cations. Kroll and Kramer (1955) reported that phosphatase activity (as determined by the release of phenol from phenyl phosphate) was unaffected by the addition of montmorillonite to soil. The failure of clay to inhibit activity suggests that phosphatases in the soil samples were already in an adsorbed form. Ramirez-Martinez and McLaren (1966b) reported that adsorbed phosphatase (acid phosphatase adsorbed on kaolinite at pH 5.4) exhibited a higher pH optimum than enzyme in solution. Activity of adsorbed phosphatase was only 25% of that of enzyme in solution. Kishk *et al.* (1976) found that preincubation of soil or substrate (methylparathion) with sterilized clays prior to assay reduced the methylparathion-hydrolyzing activity of the soil sample. The authors concluded that in both

cases adsorption of substrate by the clays reduced the reaction rate. Makboul and Ottow (1979) reported that addition of increasing amounts of calcium homoionic clay minerals (montmorillonite, illite, and kaolinite) to the assay mixture reduced the activity of acid phosphatase (from wheat seeds) at each substrate level tested. Experimental results such as those cited above support the hypothesis that adsorption of extracellular phosphatases in soil reduces their activity. Nevertheless, this conclusion may be irrelevant to the situation in soil where the reaction may take place at the surface of soil particles because of adsorption of enzyme and substrate (Gerritse and van Dijk, 1978).

There are few reports of phosphatases extracted from soil (Speir and Ross, 1978). Hayano (1977) extracted brown colored materials with phosphodiesterase activity from samples of the A horizon of a forest soil using 0.1 M phosphate (pH 7.0)-0.3 M KCl-10 mM EDTA solution. The relationship between solution volume and phosphodiesterase activity of the extract fitted an equation describing Langmuir-type desorption, suggesting that the enzymes extracted with phosphate-KCl-EDTA solution were extracellular and adsorbed to the surface of soil particles by ionic bonding. Batistic et al. (1980) extracted acid and alkaline phosphatase with other enzymes from samples of the surface horizon of a soil under permanent pasture using 0.2 M phosphate buffer

(pH 8) in the presence of 0.2 M EDTA. The extract was purified by precipitation with $(NH_4)_2SO_4$, followed by treatment with salmine sulfate to flocculate and remove humic material. The enzyme activities of the $(NH_4)_2SO_4$ and salmine sulfate preparations were fractionated by anion exchange and gel chromatography, respectively. The authors concluded that extracellular enzymes (including acid and alkaline phosphatase) extracted from soil partly occur as carbohydrate-enzyme complexes which are easily separated from humic material, and partly as humocarbohydrate-enzyme complexes. Nannipieri et al. (1980) extracted acid phosphatase and other enzymes from samples of the surface horizons of three soils using 0.14 M sodium pyrophosphate (pH 7.1). They obtained high yields of acid phosphatase; activity in the extracts was 31-66% of that in the soils. The pyrophosphate extraction employed is sufficiently mild to remove only very small amounts of enzymes from intact cells of soil microorganisms, and gives good results because it is an effective extractant of soil organic matter. Phosphatase activity, and in most cases other enzyme activities, were significantly correlated with C and organic N in the extracts of the three soils, indicating that extractants which are effective in removing C and organic N also remove enzymes. Changes in enzyme/C and enzyme/organic N ratios in the extracts over time in-

dicated that activity and organic matter were not always extracted proportionately from the three soils. Hence the enzymes extracted by pyrophosphate are not homogeneously distributed throughout organic matter in soil. These three studies indicate the variable degree of association of extracellular phosphatases with soil constituents. The evidence for carbohydrate-enzyme and humocarbohydrate-enzyme complexes, and association with organic matter in general, suggests that activity of extracellular phosphatases in situ may be modified by the microsite. Furthermore, persistence of phosphatases through association with soil constituents masks the expression of repressible-derepressible control of enzyme synthesis.

The relative contributions of enzymes in proliferating microorganisms and accumulated enzymes to soil phosphatase activity have been assessed in several studies. Jackman and Black (1952) found phytase activity to be directly proportional to the quantity of CO_2 evolved by a mixed population of microorganisms; hence they concluded that phytase activity in field soils can be accounted for on the basis of the microbial metabolism. Burangulova and Hasiev (1965) reported that phosphatase activity towards the substrate sodium phenolphthalein phosphate was increased in soil be inoculation with "phosphate bacteria," showing the importance of enzymes in proliferating microorganisms. Ramirez-

Martinez and McLaren (1966b) reported that when fungal and bacterial isolates were added to sterile irradiated soil, there was no appreciable increase in phosphatase activity after an incubation period of seven days. When sterile irradiated soil was inoculated with native soil, after seven days there was an increase in the number of bacteria and fungi, and a 47% increase in phosphatase activity. They concluded that phosphatase activity in soils reflects metabolic activities of live microorganisms, and includes a relatively stable extracellular component in soil humus. Kiss et al. (1975) concluded that accumulated phosphatase predominates over phosphatase of proliferating microorganisms in total activity of soil. Tyler (1976) suggested that the phosphatase activity in a forest soil originated from a pool of enzyme which had accumulated extracellularly. These results indicate that the relative amounts of phosphatase activity contributed by enzymes in proliferating microorganisms and accumulated enzymes will vary spatially and temporally in soil. The repressible-derepressible control of phosphatase synthesis suggests that recently synthesized enzyme is the most important source of activity in situ.

1.2 The Role of Orthophosphate in Controls on Soil Phosphatase Activity

1.2.1 Control by orthophosphate of phosphatase synthesis by microorganisms in pure culture

Controls on soil phosphatase activity operate in part upon production of microbial enzyme. Repressible-derepressible synthesis of phosphatase has been described for microorganisms in pure culture. The following examples illustrate this phenomenon. Horiuchi *et al.* (1959) proposed that a negative feedback mechanism controlled the formation of alkaline phosphatase in *Escherichia coli*, with orthophosphate acting as a repressor. Torriani (1960) studied the effect of orthophosphate on synthesis of phosphatase in the same organism, and showed that a nonspecific alkaline phosphatase (with pH optimum 8.5-9.5) was only formed in measurable amounts when orthophosphate in the medium became limiting, at which point the enzyme was formed in substantial amounts at a maximum rate. Malamy and Horecker (1961) reported that the alkaline phosphatase of *E. coli* lies outside the cell membrane, because it was liberated quantitatively into the surrounding medium when the cells were converted to protoplasts. Cashel and Freese (1964) showed that alkaline phosphatase was excreted quantitatively from intact cells of *Bacillus subtilis* following derepression of enzyme synthesis by phosphate starvation. The authors

stated that the cell wall of B. subtilis allows exit of enzymes similar to periplasmic enzymes of E. coli, and that exoenzymes are predominant in gram positive microorganisms.

Weimberg and Orton (1963) showed that the yeast Saccharomyces bisporus produced a nonspecific acid phosphatase (with pH optimum 5.5-6.0) when grown in a medium devoid of orthophosphate. Only minimal amounts of this enzyme were found in cells grown in a medium containing orthophosphate. Later Weimberg and Orton (1964) presented evidence for an exocellular site (outside the cell membrane but bound to the cell wall, as defined by the authors) for acid phosphatase of S. bisporus. They were able to prepare enzymatically active cell walls. McLellan and Lampen (1963) found that acid phosphatase was removed from the cells of Saccharomyces cerevisiae on protoplast formation, and concluded that the acid phosphatase of yeast is external to the cell membrane. Phosphatase activity of the yeast was increased by phosphate starvation. Working with the same microorganism (S. cerevisiae), Schurr and Yagil (1971) found that synthesis of acid and alkaline phosphatase was derepressed by phosphate starvation. The response of acid phosphatase (located on the outer cell wall) to regulation by orthophosphate was more marked than that of alkaline phosphatase, results which agree with those of Weimberg and Orton (1963 and 1964) and

McLellan and Lampen (1963). Weimberg (1976) investigated the relationship between the level of stored polyphosphate in growing cells of S. bisporus and repression and derepression of synthesis of acid phosphatase. Time-course studies showed no correlation between the concentration of either polyphosphate or orthophosphate in the cells, and the ability of cells to form this enzyme. The only compound investigated that was capable of repressing acid phosphatase synthesis was orthophosphate in the growth medium.

Similar control of phosphatase activity has been reported for algae. Knutsen (1968) investigated repressed and derepressed synthesis of phosphatase during synchronous growth of Chlorella pyrenoidosa. Although phosphatase synthesis was derepressed after removal of orthophosphate from the growth medium, the supply of P did not limit growth during the first few hours after removal of orthophosphate. Knutsen (1968) proposed that during the lag period immediately after transfer to the phosphate-free medium the internal pool of othophosphate was lowered to a level where derepression began. In studying extracellular phosphatases of Chlamydomonas reinhardi Patni et al. (1977) found that P-depleted cells grown on minimal orthophosphate contained and secreted both acid and alkaline phosphatase. Synthesis of alkaline phosphatase (both intra- and extra-

cellular) was almost completely repressed by increasing concentrations of orthophosphate in the growth medium, while synthesis of acid phosphatase (again both intra- and extracellular) was only partially repressed.

Regulation of repressible-derepressible synthesis of phosphatase by the solution orthophosphate concentration (external to the cell) has also been demonstrated for Neurospora crassa, Aspergillus spp., Euglena spp., and Torulopsis utilis, which with the microorganisms already mentioned represent bacteria, fungi, and algae (Schurr and Yagil, 1971). Table 2 indicates the solution concentration of orthophosphate used to repress synthesis of phosphatase in some of the experiments cited above. A comparison with estimates of the inorganic P concentration of the soil solution, 1-10 μM P (Bieleski, 1973; Bohn et al., 1979), indicates that the level of inorganic P in the soil solution would not generally repress synthesis of phosphatase. The experimental results with microorganisms grown as pure cultures in liquid media provide evidence of the general phenomenon of synthesis of a exocellular nonspecific phosphatase which is repressed by high solution concentrations of orthophosphate, and which serves the purpose of supplying the cell with phosphate in the absence of external orthophosphate and the presence of various organic phosphates. As suggested by Weimberg and Orton (1963), the kind of

Table 2. Solution concentration of orthophosphate for repression of microbial synthesis of phosphatase in liquid culture.

Reference	Microorganism and enzyme	Concentration of orthophosphate which repressed synthesis of phosphatase
Horiuchi *et al.* (1959)	*Escherichia coli* (alkaline phosphatase)	3.23 mM
Torriani (1960)	*E. coli* (alkaline phosphatase)	0.020-0.025 mM
Weimberg and Orton (1963)	*Saccharomyces bisporus* (acid phosphatase)	0.6 mM
McLellan and Lampen (1963)	*Saccharomyces cerevisiae* (acid phosphatase)	40 mM
Schurr and Yagil (1971)	*S. cerevisiae* (acid phosphatase)	0.25 mM
Patni *et al.* (1977)	*Chlamydomonas reinhardi* (alkaline phosphatase)	0.1 mM

enzyme produced relates to the physiological pattern of the particular microorganism involved.

A wide range of microorganisms in soil are capable of dephosphorylating organic phosphates of plant and microbial origin, and hence phosphatase activity towards various substrates is widespread among soil microorganisms (Cosgrove, 1967; Alexander, 1977). Regulation of repressible-derepressible synthesis of phosphatase by orthophosphate has been demonstrated for microorganisms isolated from soil. Janossy (1963) inoculated soil extracts enriched or unenriched with KH_2PO_4 with microorganisms isolated from a neutral brown forest soil, and after 48 hours separated the cells from the growth medium and dried them. Tests of powdered cells for phosphatase activity (estimated by release of phenol from disodium phenyl phosphate) indicated that increasing concentrations of KH_2PO_4 decreased enzyme activity, especially of fungi. Greenwood and Lewis (1977) demonstrated that all of five species of soil yeast of the genus Cryptococcus showed higher p-nitrophenyl phosphatase activity when grown on medium containing sodium-inositol hexaphosphate or orthophosphate-free medium than when supplied with orthophosphate. Only two species of yeast were able to grow on filter-sterilized sodium-inositol hexaphosphate as sole source of P. In considering the significance of their findings Greenwood and Lewis (1977) noted that a correlation existed

between the derepressed phosphatase pH optimum for three species and the pH of the Californian soil from which they were isolated, and suggested that the enzymes may have an ecological function. This hypothesis is supported by the range of substrates (sodium-inositol hexaphosphate, p-nitrophenyl phosphate, ß-glycerophosphate, and pyrophosphate) hydrolyzed by the three species which grew on autoclaved inositol hexaphosphate.

1.2.2 The role of orthophosphate in controls on soil phosphatase activity in incubation studies

Studies of changes in phosphatase activity of incubated soil following various nutrient additions provide information describing the average response to control by orthophosphate at the microsite. Kramer and Yerdei (1959) observed an inverse relationship between phosphatase activity and the amount of biologically utilized P in soil samples incubated with sugar and NH_4NO_3. Halstead (1964) found that addition of inorganic P prior to a nine-month incubation period did not affect soil phosphatase activity. Nannipieri *et al.* (1978) measured microbial biomass and activity during incubation of surface samples of two Dark Brown Chernozemic soils (a fine sandy loam and a clay) following amendment with various amounts of glucose, $NaNO_3$, and

KH_2PO_4. Addition of KH_2PO_4 with glucose and $NaNO_3$ produced higher initial rates of CO_2 evolution than in the treatment with only glucose and $NaNO_3$, indicating that supply of orthophosphate limited the microbial growth rate when KH_2PO_4 was not added. Addition of KH_2PO_4 with glucose and $NaNO_3$ to produce an added C : added P ratio less than or equal to 60:1 repressed synthesis of acid phosphatase in both soils. Without added KH_2PO_4 there was an increase in acid phosphatase activity, but the newly synthesized phosphatase was not very stable, especially in the sandy soil. Because phosphatase is a repressible enzyme, increases in acid phosphatase activity coincided with increases in the bacterial and fungal population (as determined by fluorescence microscopy) only in treatments without added KH_2PO_4—that is, with microbial populations growing under derepressing conditions. In a similar experiment Nannipieri *et al*. (1979) measured various indices of microbial activity and growth in incubated samples of the surface horizon of a red earth soil. Amendment of the samples with glucose and $NaNO_3$ resulted in a marked increase in the production of acid phosphatase over that of the control which received only distilled water. Much of the newly synthesized phosphatase was stabilized during the incubation period of 14 days. They also noted that acid

phosphatase activity was significantly correlated with bacterial but not with fungal biomass (as determined by fluorescence microscopy). Spiers and McGill (1979) found that acid phosphatase activity was increased up to six-fold in soil samples amended with glucose and NH_4NO_3, while synthesis of acid phosphatase by the microbial population was completely repressed by addition of KH_2PO_4 to produce an added C:added P ratio of 20:1. They concluded that the effect of orthophosphate on soil phosphatase activity is due more to its effect on enzyme synthesis than on activity of existing enzyme, a conclusion consistent with results of incubation experiments reported in the literature.

In addition to repression of enzyme synthesis, activity of existing phosphatase is strongly inhibited by orthophosphate (Speir and Ross, 1978). There are several reports of measurement of inhibition of activity of phosphatase by orthophosphate added to the assay mixture. Khaziyev (1966) reported that nuclease activity in a Chernozem was completely inhibited by 20 mM KH_2PO_4. Juma and Tabatabai (1977) found that for surface samples of three soils addition of KH_2PO_4 to the assay mixture at 25 umoles g^{-1} soil reduced acid phosphatase activity by 32, 35, and 62%, and alkaline phosphatase activity by 28 and 40%. At 2.5 umoles P g^{-1} soil inhibition was 5, 6, and 26% for acid phosphatase, and 2 and 6% for alkaline phosphatase. In a

later study with surface samples of six soils Juma and Tabatabai (1978) reported that KH_2PO_4 in the assay mixture at 10 μmoles g^{-1} soil reduced acid phosphatase activity by 21-42%, and alkaline phosphatase activity by 15-51%. At 1 μmole P g^{-1} soil inhibition was 2-21 and 3-16% for acid and alkaline phosphatase respectively. The authors also showed that inhibition of soil phosphatase activity by orthophosphate followed competitive kinetics. Spiers and McGill (1977) investigated inhibition of activity of existing enzyme in surface samples of two soils; orthophosphate in the assay mixture at 0.55 mM reduced acid phosphatase activity by 25 and 47%. At 5.5 mM P activity was reduced by 51 and 76%. Spiers and McGill (1979) concluded that because concentrations of orthophosphate in the soil solution are generally less than 0.55 mM, inhibition of activity of existing enzyme *in situ* is not usually significant. The data of Juma and Tabatabai (1977 and 1978) when expressed in terms of the orthophosphate concentration in the assay solution support this conclusion.

1.2.3 Control by orthophosphate of plant root phosphatase activity

Production of phosphatase by plant roots is also affected by the supply of orthophosphate. Rogers *et al.* (1942) reported that P-deficient corn and tomato plants showed slightly more root glycerophosphatase activity

than did plants given ample P. Nuclease activity of the roots was not affected by the previous level of P nutrition. Woolhouse (1969) stated that cell wall adenosine triphosphatase (ATP-ase) activity of roots of Agrostis tenuis is subject to two types of control by orthophosphate—endproduct inhibition, and repression of enzyme synthesis. Inhibition of ATP-ase activity became pronounced at concentrations of orthophosphate in the assay medium above 5 mM. For preparations from roots of plants grown in water culture at different levels of orthophosphate, above 10^{-2} mM P, specific phosphatase activity began to decline, and at 1 mM P, enzyme activity was only half of that from plants grown at 10^{-3} mM P. Ridge and Rovira (1971) measured the p-nitrophenyl phosphatase activity of sterile and inoculated roots of wheat plants grown at different levels of orthophosphate (1 mM, 10^{-1} mM, 10^{-3} mM, and nil). Root growth was reduced at concentrations of orthophosphate below 1 mM. Although phosphatase activity per root system was the same at all levels of P, at the lower concentrations (which produced smaller roots) the activity per unit root weight increased 2-4 times. Bieleski and Johnson (1972) reported that P-deficiency caused a 10-20 fold increase in phosphatase activity of homogenates of the duckweed Spirodela oligorrhiza. Roots of P-deficient plants posses-

sed high phosphatase activity, and the authors concluded that much of the phosphatase produced by the plant under P-deficiency is located in either the cell wall or the external membrane, for the purpose of utilizing phosphate esters released to the medium by dying plants.

1.3 The Nature and Mineralization of Organic P in Soil

1.3.1 Organic P compounds present in soil

A brief discussion of the nature of soil organic P provides a background against which studies examining controls on soil phosphatase activity towards artificial substrates must be set. Three groups of substrates can be described in the total soil organic P pool: organic P in plant and animal remains entering soil, P-containing soil organic matter, and organic P compounds contained in microbial biomass.

The organic P fraction in soil accounts for 20-80% of the total P content in surface horizons (Dalal, 1977). Halstead and McKercher (1975) in a review of the biochemistry and cycling of P reported values for organic P content of soils in the range 4-1750 μg P g^{-1} soil. The organic P content of surface horizons of some Alberta soils was reported by Dormaar and Webster (1963) to range between 170 and 630 μg P g^{-1} soil (Table 3). Variability in values for organic P content has been caused to some extent by differences in method of

Table 3. Organic P content of surface horizons of some Alberta soils (from Dormaar and Webster, 1963).

Soil zone and horizon	Total P (ppm)	Organic P* (ppm)	Organic P as % of total
Brown (Ah)	507	172	34
Dark Brown (Ah)	682	247	36
Thin Black (Ah)	1010	479	47
Black (Ah)	1220	625	51
Dark Grey (L-H)	998	415	42
Gray Wooded (L-H)	1310	500	38

* Average of values obtained with two acid-alkaline extraction methods—the Kaila-Virtanen method(successive extractions with 4 N H_2SO_4 at room temperature, 0.5 N NaOH at room temperature, and 0.5 N NaOH at 90 C), and the method of Mehta *et al*. (successive extractions with conc. HCl at 70 C for 10 minutes, 0.5 N NaOH at room temperature, and 0.5 N NaOH at 90 C). In both procedures organic P content is taken as the difference between total and inorganic P in the extract solutions.

analysis. Dormaar (1964) and Dalal (1977) stated that no method provides absolute values—that is, direct quantitative measurement is not possible. Organic P content is determined by ignition or extraction methods. With ignition methods, organic P content is taken as the difference in acid-extractable inorganic P between ignited and unignited soil samples. Ignition methods may overestimate organic P content in situations where ignition increases the solubility of the inorganic P fraction. With extraction methods, organic P content is taken as the difference between total and inorganic P in soil extracts. These methods may underestimate organic P content by failing to quantitatively extract the organic P fraction. Dormaar (1964) stated that differences in organic P content of 20 $\mu g\ P\ g^{-1}$ soil have little or no significance. Various data suggest that organic P can be either intimately bound to C, N, and S in humus, or exist as part of a pool of independent organic P compounds (Dalal, 1977). The correlation between organic C and organic P contents of the soil samples represented in Table 3 led Dormaar and Webster (1963) to conclude that in these soils organic P formed an integral part of the soil organic matter.

Halstead and McKercher (1975) and Dalal (1977) have reviewed the literature dealing with the characterization of organic P compounds present in soil. The

chemical nature of approximately half of the organic P in soil is unknown. Identified compounds include inositol phosphates, phospholipids, nucleic acids, and to a minor extent phosphoproteins, sugar phosphates (glucose-1-phosphate and glucose-6-phosphate), and glycerol phosphates. Estimation of amounts of these compounds in soil as a percentage of the total organic P content may be biased by the choice of method for determination of organic P (as described earlier), and the efficiency of the fractionation procedure.

Inositol phosphates account for up to 60% of the organic P fraction. Chromatographic studies have shown that penta- and hexaphosphates of inositol predominate. These compounds exist in complex forms bound to or associated with protein and carbohydrates. Dormaar (1967) reported values for the inositol phosphate content of surface horizons of some Alberta soils (Table 4); this fraction accounted for only a small proportion of the total organic P content of these samples (13-32%). Not all of the eluted phosphates were inositol phosphates; hence values for eluted phosphates as a percentage of organic P overestimate the contribution of the inositol phosphate fraction.

Phospholipids account for 0.5-7.0% of the total organic P content of soils. Dormaar (1970) measured the phospholipid content of Ah horizons of some Cherno-

Table 4. Inositol phosphate content of surface horizons of some Alberta soils (from Dormaar, 1967).

Soil and horizon	Total* organic P (ppm)	Organic P (% of total)	Total eluted P (ppm)	Eluted P (% of organic P) 1	2
Brown Chernozemic (Ah)	180	34	37.0	21	24
Dark Brown Chernozemic (Ah)	282	43	47.0	17	28
Thin Black Chernozemic (Ah)	401	48	74.4	19	32
Dark Gray Chernozemic (Ah)	164	29	40.8	25	
Gray Wooded (Ae)	96	47	12.2	13	

* by modified Kaila-Virtanen method: Kaila-Virtanen method described previously but preceded by two extractions—1:1 acetone, and 0.3 N NaOH.

1 organic P by modified Kaila-Virtanen method

2 organic P by method of Mehta *et al*.

zemic soils of southern Alberta (Table 5). Lipid phosphate accounted for 0.1-7.0% of the total organic P fraction in these horizons. Phosphatidylcholine and phosphatidylethanolamine make up a major part of the phospholipid component. Characterization of the fatty acids associated with the phosphate suggests that phospholipids accumulate in soils from fungal and bacterial biomass. Nucleic acids account for 5-10% of the soil organic P fraction.

The nature of the identified portion of soil organic P and the wide variety of soil microorganisms capable of hydrolyzing these compounds suggest, as a preliminary hypothesis about the persistence of organic P compounds in soil, that stability of these compounds in soil depends upon factors other than their chemical nature. Association with soil constituents may stabilize organic P compounds, or phosphatase activity towards these compounds may be inhibited _in situ_. McGill and Cole (1981) suggested that stability of organic P in soil is due primarily to reactions of the phosphate group (rather than the C moiety) with soil constituents. For example, a wide range of soil microorganisms has been shown to be capable of dephosphorylating inositol phosphates; hence hydrolysis of these compounds in soil must be limited by their low solubility in the soil solution, particularly in acid soils (Cosgrove, 1967; Alexander, 1977).

Table 5. Phospholipid content of Ah horizons of some Chernozemic soils of southern Alberta (from Dormaar, 1970).

Zone	Lipid phosphate (ppm)	Lipid phosphate* (% of organic P)
Brown	4.8-13	2.7-7.0
Dark Brown	5.8-8.0	1.9-4.4
Thin Black	0.32-12	0.1-5.4

*organic P by modified Kaila-Virtanen method

The low amounts of phospholipids and nucleic acids found in soil, the fact that these compounds account for the major part of the organic P of plant tissue (Bieleski, 1973) and animal and microbial remains (Anderson, 1967; Alexander, 1977), and the demonstrated capacity of soil microorganisms to hydrolyze these compounds suggest that phospholipids and nucleic acids are rapidly incorporated into microbial biomass (Cosgrove, 1977; Dalal, 1977).

1.3.2 Seasonal variation of organic P in soil

Another piece of evidence which suggests significant mineralization of organophosphates in soil is the seasonal variation in organic P content, particularly in the spring during the flush of plant growth. Dormaar (1972) reported seasonal variation in organic P content of unfertilized Ap horizons of two irrigated alfalfa fields on Dark Brown Chernozemic soils (Table 6). An over-winter buildup of organic P was followed by a rapid decline from April to May. Halm *et al*. (1972) observed seasonal variation in organic P content of the surface 30 cm of a Brown Chernozemic soil under native grassland (Table 7). At the same site there was also substantial variation in the level of $NaHCO_3$-extractable organic P over the growing season (Table 8). This large variation in $NaHCO_3$-extractable organic P not only is consistent with the hypothesis of mineralization

Table 6. Seasonal variation of organic P content in unfertilized Ap horizons of two irrigated alfalfa fields (from Dormaar, 1972).

Year	Organic P (ppm)*						
	Apr.	May	June	July	Aug.	Sept.	Oct.
Field 1							
1963	88	51	43	43	56	72	73
1964	242	153	146	134	116	122	138
1965	213						
Field 2							
1963	102	73	56	36	20	46	82
1964	226	158	130	110	100	120	146
1965	206						

*organic P by ignition method of Anderson (1960); organic P content is taken as the difference in inorganic P in 2 N H_2SO_4 extracts of ignited (550 C for 1 hr.) and unignited soil samples.

Table 7. Seasonal variation of soil organic P under native grassland (from Halm et al., 1972).

Date	Organic P (ppm)*
19/5/69	292 ± 30.6
20/6/69	322 ± 18.1
22/8/69	339 ± 9.8
20/9/69	336 ± 13.2
5/11/69	363 ± 8.9

* organic P by ignition method of Saunders and Williams (1955); organic P content is taken as the difference in inorganic P in 0.2 N H_2SO_4 extracts of ignited (550 C for 1 hr.) and unignited soil samples.

Table 8. Seasonal variation in $NaHCO_3$-extractable P under native grassland (from Halm et al., 1972).

Date	$NaHCO_3$-extractable P (ppm in surface 30 cm)			
	Total P	Average inorganic P	Average organic P	P_o/P_i
19/5/69	25.0 ± 0.28	4.0 ± 0.14	21.0 ± 0.31	5.3
20/6/69	23.5 ± 0.57	4.1 ± 0.07	19.3 ± 0.57	4.7
24/7/69	59.4 ± 4.90	2.3 ± 0.09	57.2 ± 4.90	24.9
22/8/69	20.1 ± 0.57	4.1 ± 0.14	16.0 ± 0.59	3.9
20/9/69	27.5 ± 1.42	2.6 ± 0.10	24.9 ± 1.42	9.6
5/11/69	29.7 ± 1.49	5.3 ± 0.43	34.4 ± 1.60	6.5
8/5/70	15.9 ± 1.34	3.6 ± 0.28	12.3 ± 1.38	3.4

over the growing season, but also suggests a role for organic P in plant nutrition. Halm et al. (1972) proposed that in cycling of P at the native grassland site mineralization of organic P is important in maintaining the balance between the transfer of P from standing dead plant material to litter to soil in a given season, and the uptake of P in new green material. Harrison (1979) observed a winter buildup of organic P in P-deficient soils of 10 woodlands.

1.3.3 Relationship of organic and available P to phosphatase activity in soil

Several workers have investigated seasonal variation of phosphatase activity in the field, and related it to changes in available and organic P. Janossy (1963) stated that field tests showed a significant negative correlation between the amount of available P and phosphatase activity in soil, and related this observation to repression of synthesis of phosphatase by orthophosphate. Khaziyev (1967) reported that the seasonal change in nuclease activity was negatively correlated with the change in ammonium-soluble organic P (considered by the author to consist predominately of nucleic compounds). Such a relationship may be masked by the production of new organic P compounds by plants and microorganisms over the season. Furthermore, a low content of readily hydrolyzable organic P (relative to

the total organic P content) may result in little seasonal change in total organic P while plant and microbial uptake of mineralized P may mask changes in available P. Saunders and Metson (1971) reported that the higher P status of soils under pasture in spring (indicated by the high uptake of P by plants in the spring flush of growth) was not due to a buildup of anion-exchangeable P or 0.01 M $CaCl_2$-soluble P in late winter and eamly spring. The authors hypothesized that rapid uptake of P by plant roots in spring was supplied by rapid release of P from organic materials. Gavrilova et al. (1973) observed seasonal variation of phosphatase activity in a pale yellow sod-podzolic soil. Large differences in activity of cropped soil over two successive years were considered to result from plant and rhizosphere production of phosphatase as influenced by the soil moisture and temperature regimes. In both years phosphatase activity increased towards the end of the growing season. Under agricultural crops, the content of acid-soluble (in 7% trichloroacetic acid) organic P peaked in the middle of the growing season, then declined towards fall; the alkaline-soluble (in 1 N NaOH) organic P content increased towards the end of the growing season, representing an increase in nucleic acid P of microbial biomass. These results support the hypothesis of increased phosphatase activity associated with increased

microbial uptake of P during decomposition of plant residues towards the end of the growing season, and a concomitant decrease in labile organic P not contained in microbial biomass.

Although reports in the literature describing the relationship of soil phosphatase activity to organic P content are variable, Gavrilova et al. (1974) reported a high positive correlation between the content of organophosphates and phosphatase activity of soils of the Belorussian SSR, and Speir and Ross (1978) concluded that most often significant positive correlations are observed. Nevertheless, the seasonal variability of both properties (as described above) indicates that correlation of values for soil organic P content and phosphatase activity which do not account for temporal variation may be invalid.

Harrison (1979) measured four P properties (total, organic, and available P contents, and phosphatase activity) in P-deficient soils of 10 woodlands, and related variation to individual woodlands, depth in the soil profils, humus type, and season. Although each property varied widely, available P content and phosphatase activity were more variable than total and organic P contents. Phosphatase activity together with available and organic P contents showed seasonal variation in the surface horizon. While phosphatase activity was

highest in the winter months and lowest in summer, available P showed the opposite pattern—it was lowest in winter and highest in summer. In the P-deficient soils high phosphatase activity was associated with low total and organic P contents (expressed as μg cm^{-3} soil). The latter two observations fit the hypothesis that increased phosphatase activity associated with low levels of available P represents derepression of phosphatase synthesis by low levels of orthophosphate *in situ*.

Harrison and Pearce (1979) measured seasonal variation of phosphatase activity over 14 consecutive months in surface samples of 48 woodland soils; 6 and 21% of the total variation in phosphatase activity was seasonal when activity was expressed as phenol liberated g^{-1} soil and cm^{-3} soil, respectively. No seasonal pattern was detectable within individual woodlands as much of the variation within each woodland site over the sampling period was spatial rather than temporal. When data for all 48 sites were averaged a seasonal pattern for phosphatase activity expressed as phenol liberated g^{-1} soil was identified; activity gradually increased from May to December, then declined. When phosphatase activity was expressed as phenol liberated cm^{-3} soil, two peaks occurred. This second pattern was explained by Harrison and Pearce (1979) as

follows: the summer peak of activity from May to October resulted from production of enzyme by roots and microorganisms during the active plant growth; the winter peak of activity from October to March was associated with accumulation and decomposition of leaf-litter on the soil surface (litter was shown to contain high phosphatase activity). In both cases maximum activity occurred in winter, and the seasonal pattern of activity did not resemble that of available P, results similar to those reported in the previous study. When the same data were adjusted to field temperature, the seasonal pattern of phosphatase activity expressed as phenol liberated cm^{-3} soil showed maximum activity in summer. Furthermore, the seasonal pattern of phosphatase activity adjusted to field temperature was similar to the seasonal pattern of available P. Because measurements of phosphatase activity represent level of enzyme and hence potential activity, and not activity *in situ*, such adjustment of activity measurements to represent ecologically significant activity is suspect.

The relationship between phosphatase activity and available P is not always defined by repressible-derepressible control of enzyme synthesis. For example, Spiers and McGill (1979) compared surface samples of four soils, and observed little relationship between extractable P and phosphatase activity. Organic C

content appeared to be more closely related to phosphatase activity.

1.3.4 Utilization of organic P by plants

Further evidence for the significance of mineralization reactions in soil comes from numerous studies of plant response to organic P fertilizers. McKercher and Tollefson (1978) studied the response of barley (Hordeum vulgare) to P from nucleic acids and phospholipids in a P-deficient soil. Comparison of values for plant biomass and tissue P concentration showed deoxyribonucleic acid (DNA) and KH_2PO_4 at 50 ug P g^{-1} soil to be similarly efficient in supplying P to the plants. Measurements of $NaHCO_3$-extractable inorganic P showed nearly equal amounts of available P from DNA and KH_2PO_4 after five days. Phosphatidylcholine was mineralized less rapidly than DNA, and was an inferior source of P compared with KH_2PO_4. Other studies have also shown that plants can use organic P compounds as the sole source of P under sterile and nonsterile conditions via root production of phosphatases (Rogers et al., 1942; Szember, 1960; Estermann and McLaren, 1961; Sen Gupta and Cornfield, 1967). Dalal (1977) concluded that while plants can use glycerophosphate, sugar phosphates, nucleic acids, nucleotides, inositol hexaphosphate, and lecithin as sources of P, there is no unequivocal evidence that plants utilize organic P from

the soil solution. Nevertheless, some of the organic P extracted from soil by Wild and Oke (1966) was shown to be available to plants.

Various studies have investigated the effect of plant growth on levels of organic P in soil and its relationship to phosphatase activity of the rhizosphere. Sekhon and Black (1968) reported that P uptake by plants was significantly correlated with organic P mineralized during incubation of soil samples in the laboratory, independently of CO_2 evolved and labile inorganic P. Their results support the hypothesis that some of the P taken up by plants was initially present as organic P and was mineralized by soil microorganisms during plant growth. In another experiment, Sekhon and Black (1969) demonstrated that in surface samples of six soils organic P extractable in 1 N HCl followed by 0.5 N NH_4OH was significantly lower in soil samples planted to four successive crops of sorghum and oats (when roots were removed from the soil prior to analysis) than in control soil samples and uncropped soil samples incubated in a moist condition. While in uncropped soil increases in extractable inorganic P were similar to decreases in extractable organic P, in cropped soil decreases in extractable organic P were much greater than increases in extractable inorganic P because of uptake of P by the plants.

Further evidence that soil organic P may be decreased by growing plants has been presented by Thompson and Black (1970a). Growth of five successive corn seedlings (each for one week) in 30 ml of water in the presence of 0.5 g of soil contained in an envelope of Teflon-coated glass fiber filter paper decreased the extractable organic P content of the soil (after correction for organic P contributed by plants) relative to the amount without plants present. In the simulated rhizospheres plant production of organic P compounds and plant-induced disappearance of native organic P occurred simultaneously. Furthermore, the plant-induced decrease in extractable organic P occurred without physical contact between soil and roots. Thompson and Black (1970b) tested the hypothesis that such decreases in organic P result from an increased rate of mineralization of soil organic P due to increased phosphatase activity in the presence of plants. Phosphatase preparations from solutions in which corn seedlings had been grown, and purified phosphatase were incubated with soil in the absence of plants. The soil used was previously shown to undergo a decrease in native organic P in the presence of corn plants. Additions of phosphatase resulted in an increase in organic P instead of the decrease predicted by the phosphatase hypothesis. The authors suggested that P immobilized in the biomass of microorganisms

which grew on added phosphatase and associated organic materials exceeded any mineralization of native soil organic P. Furthermore, deficiency in technique (in addition of active phosphatase and precise measurement of the sensitive organic P fraction) may have affected the results. These results also suggest enzymes active in situ are associated with a proliferating derepressed microbial population.

Neal (1973) investigated the influence of selected grasses and forbs on soil phosphatase activity. Each species was grown separately in the greenhouse for 10 weeks; soil was then separated from the plant roots, sieved, and stored at 4 C until analysis for enzyme activity. Acid phosphatase activity was not altered by grass species classified as dominant, co-dominant, or increasers; plant species classified as invaders, however, produced a statistically significant increase in acid phosphatase activity. Although there was no difference in $NaHCO_3$-extractable P measurements before seeding and after harvest, Neal (1973) suggested that increased phosphatase activity under invader species reflects a potentially higher P uptake. His results may explain the slight-to-nil response to fertilizer P of grazed grasslands, even when the reserve of soil P (by soil test) is low.

Boero and Thien (1979) investigated the effect of corn roots on P availability in a macrorhizosphere (in

which the entire root system was confined to a core 2 cm in diameter) using three soils. After three weeks growth acid phosphatase was enriched by 17, 20, and 40% in the macrorhizosphere, and to a lesser extent 5 mm away from the central core. In the three soils the degree of enrichment was inversely proportional to residual phosphatase activity, organic matter content, and clay content. Although available P (extracted with 0.03 N NH_4F in 0.025 N HCl) averaged for the three soils was depleted by 11 and 16% after 2 and 3 weeks of growth respectively, the changes were not statistically significant. Furthermore, there was no uniform decrease in organic P in the macrorhizosphere; hence the authors concluded that enzyme activity was not limiting mineralization of phosphatase-hydrolyzable compounds. The ignition method used to determine organic P, however, may not be sensitive enough to detect changes in the phosphatase-hydrolyzable organic P fraction.

Although evidence has been presented in support of the hypothesis that plant roots produce phosphatases to utilize organic P compounds in soil, it is not conclusive. Alexander and Hardy (1981) interpreted the inverse relationship between acid phosphatase activity of mycorrhizal roots of Sitka spruce and the level of 0.5 N acetic acid-extractable P of decaying organic matter about the roots as derepression of phosphatase synthesis,

but they did not obtain evidence of plant utilization of soil organic P. Continuing poor growth of trees at the site was largely attributable to P-deficiency, indicating that high phosphatase activity of mycorrhizal roots did not compensate for low levels of labile inorganic P. The authors suggested that higher phosphatase activity may result from a greater proportion of senescing root tissue associated with a higher turnover of absorbing roots on P-deficient sites.

1.4 The Relationship of Phosphatase Activity to Other Soil Properties and Cultural Practices

1.4.1 Organic C content

In most cases phosphatase activity is directly related to organic C content in soil profiles and in different soils (Speir and Ross, 1978). For example, Speir (1977) found that acid phosphatase activity in eight topsoils from tussock grasslands was correlated highly significantly with organic C, and Juma and Tabatabai (1978) reported that acid and alkaline phosphatase activity were significantly correlated with organic C in pooled data of five soil profiles. Because of its relationship to organic C, phosphatase activity decreases with depth in the soil profile (Juma and Tabatabai, 1978; Speir and Ross, 1978).

1.4.2 Soil pH

The relationship between phosphatase activity and soil pH is such that the larger the difference between the natural soil pH and the optimum pH for enzyme activity, the lower the activity *in situ* (Speir and Ross, 1978). Furthermore, Speir (1977) found that acid (pH 6.3) phosphatase activity was correlated highly significantly and negatively with pH in nine surface horizons of a climosequence of soils in tussock grasslands. Juma and Tabatabai (1978) reported that acid (pH 6.5) phosphatase activity was significantly and negatively correlated, while alkaline (pH 11.0) phosphatase activity was significantly and positively correlated, with the pH of surface samples of 11 soils studied.

1.4.3 P fertilization

The effect of available P content and inorganic P fertilizers on the level of phosphatase activity in soil is variable, particularly when viewed at a level of resolution which regards measurable changes in the field. Controls on phosphatase activity via repression of enzyme synthesis or inhibition of existing activity by orthophosphate operate at the microsite in which the enzyme is active. While soil available P status affects phosphatase activity through such controls, it also has an indirect effect by influencing plant

productivity and hence amounts of enzyme in the system. Burangulova and Hasiev (1965) found that soil phosphatase activity was decreased by application of inorganic P fertilizers. Skujins (1967) concluded that because phosphatase activity in soil is inversely proportional to the amount of biologically available P, addition of inorganic P fertilizers usually diminishes it. Chunderova and Zubets (1969) reported that in 544 derno-podzolic soils phosphatase activity increased as application of fertilizer P increased the soluble P content up to 20 mg 100 g^{-1} soil; phosphatase activity decreased at higher levels of soluble P, and disappeared completely at soluble P levels of 60-80 mg 100 g^{-1} soil. Speir and Ross (1978) suggested that low levels of fertilizer P increase phosphatase activity indirectly by increasing plant productivity and microbial activity, hence soil organic matter content and enzyme level. Decreased phosphatase activity from high levels of fertilizer P results from repression of enzyme synthesis and inhibition of existing activity by orthophosphate. Their hypothesis is supported by the results of Spiers and McGill (1979); in a Black Chernozemic soil with high organic matter content and high initial phosphatase activity, P fertilization at 27 or 54 kg P ha^{-1} yr^{-1} for 5 years reduced phosphatase activity by about 20%. In a Gray Luvisolic soil with low

organic matter content and low initial phosphatase activity, P fertilization at 54 kg P ha^{-1} yr^{-1} for 5 years tended to increase activity.

1.4.4 Microbial numbers and plant productivity

The relationship between microbial numbers and soil phosphatase activity in the field is not well-defined. While Kramer and Yerdei (1959) reported no direct relationship between phosphatase activity and total microbial activity (as evidenced by amount of sugar consumed during composting), Geller and Dobrotvors'ka (1960) found that in Ukranian podzolized gray soils phosphatase activity in rhizospheres of winter wheat, clover, and lucerne was closely related to microbial activity. Ramirez-Martinez and McLaren (1966b) reported no correlation between changes in numbers of bacteria and fungi, and changes in phosphatase activity in soil over several sampling dates. In spite of variable results, sufficient evidence has been presented to discount the hypothesis that phosphatase activity may be used as an index of microbial activity in soil. Because of repressible-derepressible synthesis of phosphatase by soil microorganisms, a positive correlation between microbial numbers and phosphatase activity is only expected for microbial populations growing under derepressing conditions (Nannipieri et al., 1978). The results of Geller and Dobrotvors'ka

(1960) suggest that availability of substrate for microbial growth and demand for orthophosphate by plant roots and soil microorganisms in the rhizosphere may promote derepressing conditions.

In situations where fertilizer application or rotation of crops increases plant production, soil organic matter content, and microbial activity, levels of phosphatase in soil are usually increased. Hence non-P fertilizers and organic fertilizers generally increase soil phosphatase activity (Vlasyuk et al., 1957; Geller and Dobrotvors'ka, 1960; Burangulova and Hasiev, 1965; Speir and Ross, 1978). Khan (1970) found greater phosphatase activity and total microbiological activity (by measurement of dehydrogenase activity) in a five-year rotation of grains and legumes than in a wheat-fallow sequence on a Gray Luvisolic soil. Both activities were probably related to soil organic matter content, which was higher for the five-year rotation. Moreover, certain plant species may increase levels of phosphatase in soil more than others, as Neal (1973) demonstrated for plants growing on over-grazed grassland sites in Alberta.

CHAPTER 2

CHARACTERIZATION OF PHOSPHATASE ACTIVITY IN SURFACE SAMPLES OF TWO SOILS

2.1 Introduction

2.1.1 Michaelis-Menten kinetics

Application of Michaelis-Menten kinetics to phosphatase activity in soil has provided information about the physical location and state of enzyme activity in soil, and about controls on activity. It has also provided information consistent with the hypothesis that the activity is enzymatic in nature. This theory assumes (for a single substrate, single enzyme reaction) that the enzyme reacts with the substrate in two steps, and that the reaction is reversible:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E + P$$

where E represents uncombined enzyme, ES represents enzyme-substrate complex, E_t represents all the enzyme present, S represents substrate, and P represents

product. Derivation of the Michaelis-Menten equation, which is the rate equation for an enzyme-catalyzed reaction involving a single substrate (in the context of this discussion), is based on two assumptions: (i) the substrate concentration is so high that the enzyme is nearly saturated with substrate (that is, virtually all the enzyme in the system is present in the enzyme-substrate complex), and (ii) a steady-state concentration of enzyme-substrate complex is maintained. The Michaelis-Menten equation

$$v_o = \frac{V_{max}\,[S]}{K_m + [S]}$$

relates initial reaction velocity (v_o), maximum velocity (V_{max}) which is equal to $k_{+2}\,[E_t]$, and initial substrate concentration ($[S]$) through the Michaelis-Menten constant (K_m), where

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

The value of K_m is equal to the substrate concentration at which the activity is half maximal.

Values for K_m and V_{max} are obtained experimentally by measurement of the initial reaction velocity at various substrate concentrations while holding the concentration of enzyme (amount of soil in the assay mixture) constant. When data are fitted to a linear transformation of the Michaelis-Menten equation, values of K_m and V_{max} can be obtained by simple linear regression analysis. Various values have been reported for phosphatase activity of soil samples or materials extracted from soil (Table 9). Differences in values reported in Table 9 partly result from differences in the assay methods used.

2.1.2 Discrepancies in the fit of Michaelis-Menten kinetics to soil phosphatase activity measurements

Discrepancies in the fit of Michaelis-Menten kinetic theory for solutions to experimental data obtained from batch-type assays also provide information about the effect of the microenvironment on stabilized phosphatase activity in soil samples. Cervelli *et al.* (1973) determined K_m values for soil phosphatase activity by a method which corrected the values to account for adsorption of substrate. They suggested that the action of phosphatases in soil follows two steps,

Table 9. Values of K_m and V_{max} for phosphatase activity of soil samples or materials extracted from soil.*

Reference	Phosphatase activity	K_m (mM)	V_{max}** (µmoles h^{-1} g^{-1} soil)
Tabatabai and Bremner (1971)	acid	1.26-4.58	0.83-4.87
Cervelli et al. (1973)	acid	9.7-16.4 0.35-5.40***	
Gerritse and van Dijk (1978)	acid alkaline	0.05-0.20 0.50-1.00	
Batistic et al. (1980)	acid+ alkaline+	9.2 17.5	0.083++ 0.019++
Eivazi and Tabatabai (1977)	acid alkaline diesterase	1.11-3.40 0.44-4.94 0.25-1.25	1.44-4.49 0.89-4.23 0.33-0.91
Browman and Tabatabai (1978)	diesterase	1.26-2.02	0.37-3.81
Kishk et al. (1976)	triesterase+++	0.125 0.50	0.056 0.100

* differences in values partly result from differences in assay methods

** µmoles p-nitrophenol h^{-1} g^{-1} soil unless otherwise stated

*** values corrected for substrate adsorption

\+ extracted and purified enzymes

++ µmoles p-nitrophenol min^{-1} mg^{-1} protein

+++ substrate was methylparathion; values reported represent two extremes of a nonlinear plot

$$S_a \rightleftharpoons S_s$$

$$E + S_s \rightleftharpoons ES_s \longrightarrow E + P$$

where S_a represents adsorbed substrate, S_s represents substrate in solution, and the other symbols have the meanings previously described. Although Cervelli et al. (1973) hypothesized that the reaction takes place in solution, Gerritse and van Dijk (1978) suggested that the phosphatase reaction may take place mainly at the surface of soil particles because of adsorption of both enzyme and substrate. Cervelli et al. (1973) stated that the substrate p-nitrophenyl phosphate was adsorbed according to the Freundlich isotherm,

$$x/m = KC^{1/n}$$

where x represents the mass of substrate taken up by mass m of soil, C the concentration of substrate in solution at equilibrium, and K and n are experimentally determined constants depending on adsorbent, adsorbate, solvent, and temperature. Using the Lineweaver-Burk transformation, the authors derived an equation for

determination of the Michaelis-Menten constant in which the solution concentration of substrate is calculated from the Freundlich isotherm:

$$\frac{1}{v_o} = \frac{K^n K_m}{V_{max}} \left(\frac{1}{x/m} \right)^n + \frac{1}{V_{max}}$$

They obtained K_m values ranging from 9.7-16.4 mM when substrate adsorption was not taken into account; when adsorption was accounted for, the corrected K_m values (K_{mc} values) ranged from 0.35-5.4 mM. The K_{mc} values are smaller than those for K_m because in heterogeneous systems such as soil not all the substrate is available to react with the enzyme, and hence more substrate is necessary to have the same reaction rate as in solution. The K_{mc} values are close to the values of K_m reported for acid phosphatases from plants and microorganisms.

Brams and McLaren (1974) showed that in the substrate range 0.1-1.0 mM Michaelis-Menten kinetics adequately described the concentration of substrate and product leached from columns of soil crumbs as a function of the initial substrate concentration, phosphatase activity of the soil, column length, and flow

rate. They also obtained evidence that hydrolysis of substrate in the columns was diffusion-limited.

Irving and Cosgrove (1976) reported that an Eadie-Hofstee plot (v_o, the initial reaction rate, versus $v_o/[S]$, where $[S]$ is the substrate concentration) of experimental data indicated that p-nitrophenyl phosphatase activity of a kraznozem did not follow Michaelis-Menten kinetics for solutions. In their work both substrate and product were measured spectrophotometrically during the course of hydrolysis of p-nitrophenyl phosphate by the soil sample. Substrate adsorption was accounted for; the quantity of substrate sorbed (S_{sorbed}) during the assay was calculated as the difference between the quantity of substrate added (S_{added}), and the sum of the quantity present in the outer solution ($S_{sol.}$) and the quantity hydrolyzed at time t (P).

$$S_{sorbed} = S_{added} - (S_{sol.} + P)$$

The plot of v_o versus $v_o/[S]$ was nonlinear, and hence could not be used to calculate K_m directly. A value for V_{max} was estimated by extrapolation from the Eadie-Hofstee plot and used to obtain an apparent K_m value from the plot of v_o versus $[S]$, based on the definition of K_m as the substrate concentration at which activity is

half maximal. They determined apparent K_m values using two different amounts of soil in the assay mixture (Table 10).

Progressively increasing the concentration of orthophosphate in the assay solution progressively decreased the quantity of substrate sorbed by the soil sample, suggesting that p-nitrophenyl phosphate was adsorbed by the soil through its phosphate group.

Irving and Cosgrove (1976) considered the deviation from Michaelis-Menten kinetics to result from the nature of the activity itself, or from the nature of the microenvironment in which the activity is located. If p-nitrophenyl phosphatase activity in soil behaves like that of a membrane-bound enzyme, several microenvironmental effects could be responsible for the deviation, involving diffusion of substrate through the unstirred solution surrounding the soil matrix, substrate diffusion through pores in the soil matrix to the enzyme, and electrostatic effects between charged substrate ions and fixed charges of the soil matrix.

Product formation followed zero-order kinetics for at least the initial 40 minutes of the reaction. Irving and Cosgrove (1976) hypothesized that substrate concentration in the region of the enzyme was maintained at a constant level during this period by transfer from other substrate pools, particularly the substrate in the

Table 10. Apparent K_m and V_{max} values for p-nitrophenyl phosphatase activity of a kraznozem (from Irving and Cosgrove, 1976).

Amount of soil in assay mixture (g)	V_{max}* (nmol. min^{-1})	K_m** (mM)
0.5	87	1.5
0.1	19.6	0.7

* estimated by extrapolation from the nonlinear Eadie-Hofstee plot.

** estimated from the plot of v_o versus [S], based on definition of K_m as the substrate concentration at which activity is half maximal.

bulk solution and that sorbed by the soil. They proposed a model (Figure 3) to describe this hypothesis in which S_o^- represents substrate in the outer solution, S_i^- represents substrate in the vicinity of the enzyme (in the inner solution), AS represents sorbed or relatively nondiffusible substrate, and A^+ represents the sorption sites of the soil matrix. Other symbols have the meanings described previously. Reaction 1 and 4 maintain the concentration of substrate in the inner solution as it is reduced by hydrolysis. The authors proposed that step 1 could be regarded as a Donnan equilibrium, and step 4 as a solubility product relationship or sorption equilibrium. Inorganic P produced by hydrolysis displaces sorbed substrate into the inner solution, tending to maintain the substrate concentration in the inner solution at a constant level.

The discrepancies in fit of Michaelis-Menten kinetics for solutions to measurements of phosphatase activity of soil as reported by Cervelli *et al.* (1973) and Irving and Cosgrove (1976) indicate that the phosphatase reaction is affected by the solid phase in the assay mixture. Because reaction rate is affected by the influence of microenvironment on access of substrate to enzyme, Michaelis-Menten kinetics do not account for all

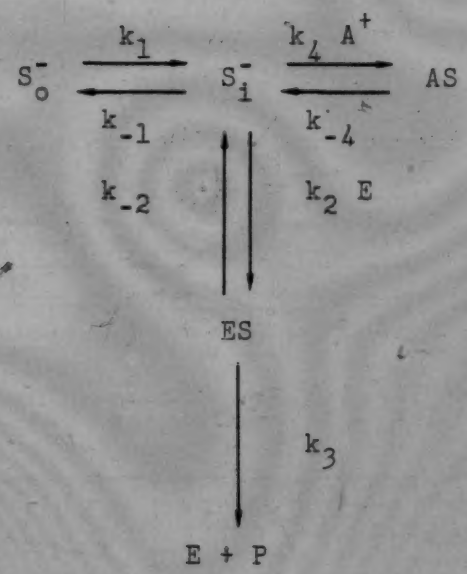


Figure 3. Mechanism for hydrolysis of of p-nitrophenyl phosphate in in a kraznozem, in which the concentration of substrate in the vicinity of the enzyme (S_i^-) is maintained by transfer from two other substrate pools—substrate in the outer solution (S_o^-), and substrate sorbed by the soil (AS) (from Irving and Cosgrove, 1976).

the factors which control phosphatase activity *in situ*.

2.1.3 Variation in K_m values

The application of Michaelis-Menten kinetics to phosphatase activity in soil has shown that microenvironment affects reaction rate. Determination of K_m values for soil samples in batch-type assays provides an apparent K_m value which is a composite of values for the various components of activity. This concept is consistent with results obtained by Paulson and Kurtz (1970) in measurement of K_m for soil urease activity. The value of K_m changed with fluctuations in the microbial population. Regression of urease activity on the number of ureolytic microorganisms was used to partition total activity between an adsorbed and microbial component. The partitioned urease activities were then used to calculate K_m values of 0.057 and 0.252 M for the microbial and adsorbed forms, respectively. The authors emphasized that the measured changes in value of K_m for the soil as a whole indicated a change in the environment of the urease molecules in the soil, which was caused by a shift in the proportions of two components of activity located in two different microenvironments. Similarly, Kishk *et al.* (1976) obtained two arbitrary K_m values (0.125 and 0.50 mM) for the hydrolytic activity of soil samples towards methylparathion by extrapolating the two extremes of a nonlinear Lineweaver-

Burk plot. They suggested that the two values may indicate more than one source of methylparathion hydrolase in soil, or may as two extremes, pertain to enzymes in the free state (0.125 mM) and those in the highest state of adsorption (0.50 mM). The higher value would then represent an apparent constant which is affected by substrate distribution between the solid and solution phases.

Juma and Tabatabai (1978) concluded that variation in K_m values for phosphatase activity in soils is partly due to variation in the concentration of soluble native orthophosphate in soils because orthophosphate is a competitive inhibitor of acid and alkaline phosphatase. Competitive inhibitors alter the value of K_m but not V_{max}.

Makboul and Ottow (1979) investigated the influence of calcium homoionic clay minerals (montmorillonite, illite, and kaolinite) added to the assay mixture on K_m values of wheat seed acid phosphatase. The value of K_m increased with the amount of clay mineral added (Table 11), indicating that affinity of enzyme for substrate was reduced by adsorption of substrate or enzyme to the clay. Similarly, variation in the extent of adsorption of enzyme or substrate may partly explain variation in K_m values reported for phosphatase activity of soil samples.

Table 11. Effect of increasing amounts of three clay minerals in the assay mixture on values of K_m for wheat seed acid phosphatase (from Makboul and Ottow, 1979).

Clay mineral	Amount of* clay added (mg)	K_m (mM)
none	-	1.43
montmorillonite	10	24.0
	20	50.0
	30	82.3
illite	10	2.65
	20	6.94
	30	7.65
kaolinite	10	4.23
	20	5.54
	30	8.02

* mg clay ml^{-1} assay solution containing 0.04 mg enzyme ml^{-1}

2.1.4 Conclusions and experimental objectives

Studies of the nature of phosphatase activity in soil indicate that activity of a soil sample represents the contribution of proliferating microorganisms, and a stable extracellular component. Both components must be considered in studies of controls on activity, and characteristics of phosphatase in soil. Moreover, measurement of phosphatase activity yields a value which is in most cases an index of potential activity in situ. Kinetic constants determined without extraction of phosphatases from the soil matrix are averages of values associated with the various components of activity in soil, whose relative contributions to the whole vary spatially and temporally in soil. Variation in K_m values reported for soil samples and discrepancies in fit of Michaelis-Menten kinetics to measurements of soil phosphatase activity partly result from the influence of the microenvironment on accessibility of substrate to enzyme.

The objective of this initial study was to characterize the phosphatase activity of air-dry samples of the surface horizons of two soils by determination of pH optima, and values of K_m and V_{max}. The first hypothesis tested was that phosphatase activity in the soil samples can be resolved into an acid and alkaline component on the basis of measured pH optima, with either or both present. Determination of pH optima provides indirect

information about the source of enzyme in the particular soil studied. The second hypothesis tested was that soil phosphatase activity (measured in batch-type assays) can be adequately described by application of Michaelis-Menten kinetics, yielding apparent K_m and V_{max} values. Such kinetic treatment provides indirect information about the physical location and state of phosphatase in soil, and indicates whether the micro-environment in which the enzyme is located affects its activity.

2.2 Materials and Methods

2.2.1 Soils

The soil samples used in this study were air-dry (less than 2 mm) samples of the surface (Ap) horizon of the Malmo SiCL (under barley stubble) and the Breton L-SiL (under hay and unfertilized). Some characteristics of these soil samples are described in Table 12.

2.2.2 Phosphatase activity measurements

The method used for assay of phosphatase activity was a modified form of that of Tabatabai and Bremner (1969), which involves colorimetric determination of p-nitrophenol released by phosphatase activity of soil samples during incubation with buffered p-nitrophenyl phosphate solution and toluene at 37 C for 1 hour. Soil (1.0 g, air-dry basis) was weighed into a 50-ml Erlen-

Table 12. Selected properties of surface samples (less than 2 mm, air-dry samples of the Ap horizon) of two soils used in this study.

Property	Malmo soil (Black Chernozemic)	Breton soil (Gray Luvisolic)
Texture*	SiCL	L
pH - in H_2O	5.9	6.3
- in 0.01 M $CaCl_2$	5.3	5.8
Organic C (%)**	5.9	1.4
Total P (ppm)+	1180 ± 12	501 ± 11
$NaHCO_3$-extractable P (ppm)++		
- inorganic P	14.9 ± 0.84	7.3 ± 0.57
- organic P	19.7 ± 1.4	5.5 ± 0.79

* from Alberta Soil Survey Reports 21 and 24

** by Leco induction furnace

+ by method of Parkinson and Allen (1975), omitting use of selenium

++ by method described in chapter 3

meyer flask; after addition of 4 ml of buffer solution (Modified Universal Buffer of Skujins *et al.*, 1962), 0.25 ml of toluene, and 1 ml of 0.114 M sodium p-nitrophenyl phosphate solution (made up in buffer of appropriate pH), the flask was swirled to mix the contents, stoppered, and placed into a shaker contained in a water-bath maintained at 37 C. After 1 hour the flask was removed and unstoppered; 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added to the flask; the contents were mixed by swirling, and then filtered through Whatman No. 2 filter paper. Aliquots of filtrate were diluted in appropriate buffer, and the absorbance measured at 400 nm. The concentration of p-nitrophenol in the filtrate was determined by comparison with a calibration curve obtained for standard solutions of p-nitrophenol (0, 2, 4, 6, 8, and 10 $\mu g\ ml^{-1}$) in buffer. A control in which the substrate solution was added to the flask after addition of $CaCl_2$ and NaOH (prior to filtration) was employed to account for yellow color resulting from soil organic matter, and p-nitrophenol contained in the substrate solution, and not from hydrolysis of substrate.

Results obtained were expressed as μmoles p-nitrophenol $h^{-1}\ g^{-1}$ soil (oven-dry basis). In determination of pH optima, 5, 10, or 20 replicates were used, depending upon variability of the phosphatase activity measurements. The raw data describing the relationship between phos-

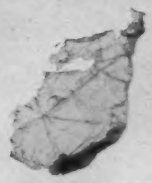
phatase activity and buffer pH for both soils (to accompany Figures 4 and 5) are presented on pages 104-5.

2.2.3 Determination of kinetic constants

A procedure similar to that of Tabatabai and Bremner (1971) was used for kinetic measurements. The values of K_m and V_{max} were calculated by simple linear regression from the Hanes-Woolf transformation of the Michaelis-Menten equation using data obtained in a series of assays in which the initial substrate concentration was varied while holding the quantity of enzyme constant. The Hanes-Woolf transformation of the Michaelis-Menten equation,

$$\frac{[S]}{v_o} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot [S]$$

where the symbols have the meanings described previously, produces a linear plot of $[S]/v_o$ against $[S]$ in which the slope has the value of $1/V_{max}$, and the intercept on $[S]/v_o$ has the value of K_m/V_{max}. The slope of the line is equal to the regression coefficient (b) of $[S]/v_o$ on $[S]$, which can be calculated by simple linear regression. If $[S]$ and $[S]/v_o$ are represented by X and Y respectively,



$$b_{yx} = \frac{\Sigma XY - \frac{\Sigma X \, \Sigma Y}{n}}{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}$$

The value of b can be used with the mean values of $[S]/v_o$ and $[S]$ to calculate the value of the intercept on $[S]/v_o$ (a).

$$a = \overline{Y} - b\overline{X}$$

The values of K_m and V_{max} can then be calculated from the Hanes-Woolf transformation using the values obtained for the regression coefficient (b) and the intercept on $[S]/v_o$ (a).

$$b = 1/V_{max} \qquad V_{max} = 1/b$$

$$a = K_m/V_{max} \qquad K_m = a \cdot V_{max}$$

Substrate solution concentrations used were 2.5, 5, 25, 50, 75, and 100 mM, and 1, 5, 25, 50, 75, and 100 mM for the Malmo and Breton samples respectively. The corresponding concentrations of substrate in the assay solution (resulting from dilution of 1 ml of substrate solution by 4 ml of buffer in the assay mixture) were 0.5, 1, 5, 10, 15, and 20 mM, and 0.2, 1, 5,

10, 15, and 20 mM, respectively. The kinetic measurements were carried out at the pHoptima of the two soils, pH 8.0 for the Malmo samples, and pH 6.0 for the Breton samples. Phosphatase activity was determined in triplicate as described above, with shaking during the incubation period. The calculation of K_m and V_{max} from the Hanes-Woolf transformation of the Michaelis-Menten equation is further described on pages 106-9.

The data obtained for the two soils were also plotted according to the Eadie-Hofstee transformation (v_o against $v_o/[S]$) of the Michaelis - Menten equation, where

$$v_o = -K_m \cdot \frac{v_o}{[S]} + V_{max}$$

to test for departures from linearity that are not apparent with the Hanes-Woolf transformation.

2.3 Results

2.3.1 pH optima

The phosphatase activity of air-dry samples of the Ap horizon of the Malmo and Breton soils varied over the pH values tested (Figures 4 and 5). Numbers along the abscissa in the figures represent buffer pH, not the pH of the solution at the microsite where the enzyme is located. The curve for the Malmo samples (Figure 4)

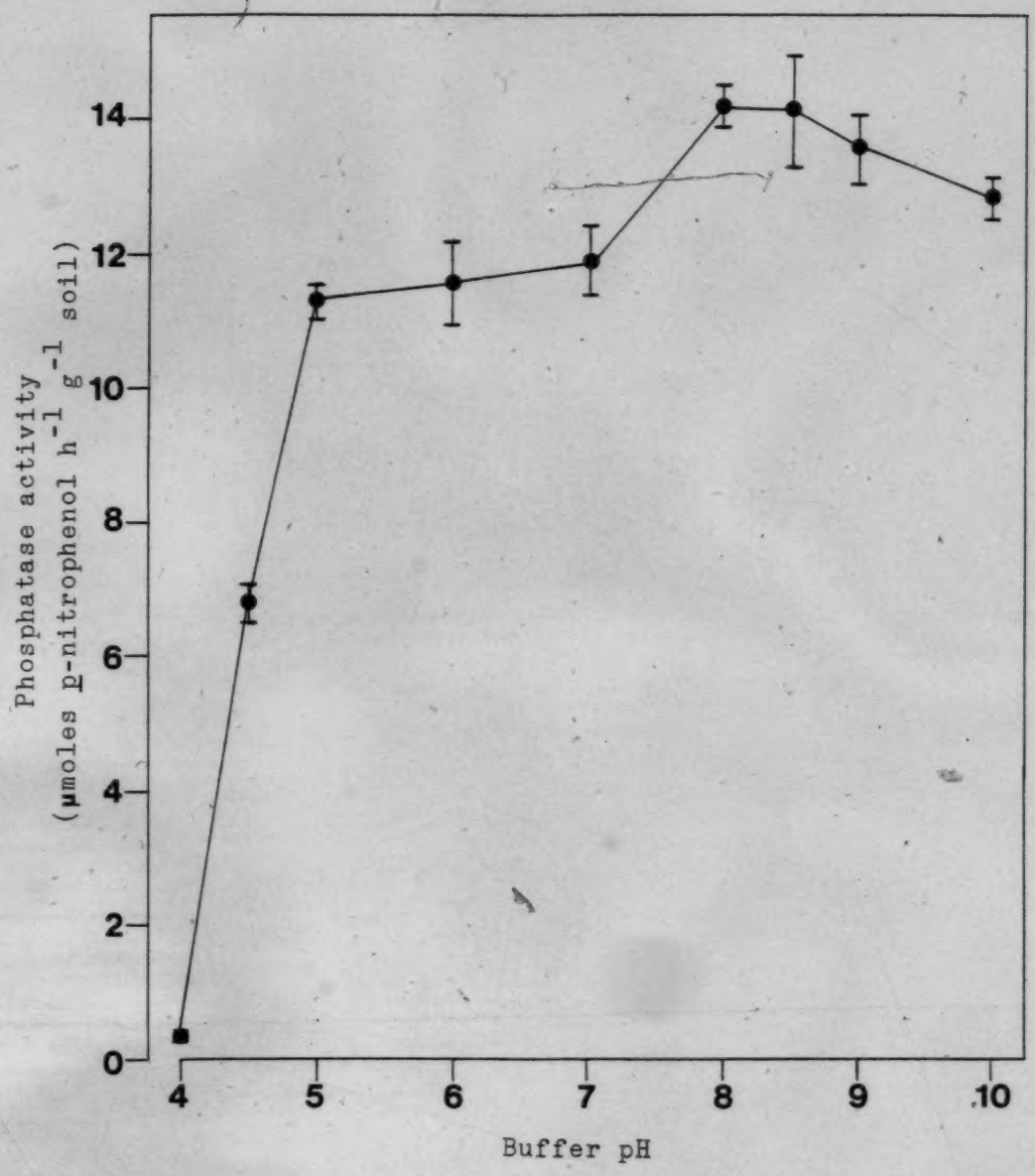


Figure 4. Relationship between phosphatase activity and pH for air-dry samples of the surface horizon of the Malmo SiCL; vertical bars represent one standard deviation.

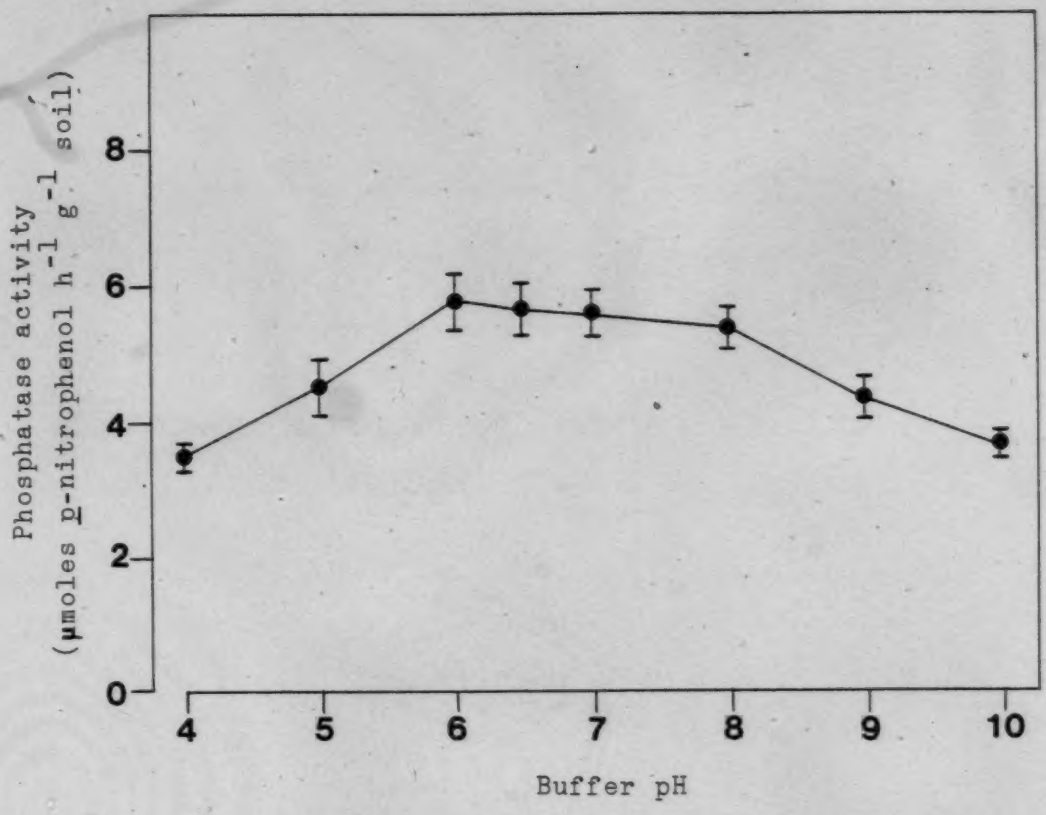


Figure 5. Relationship between phosphatase activity and pH for air-dry samples of the surface horizon of the Breton L-SiL; vertical bars represent one standard deviation.

shows optimum activity at pH 8.0-9.0, with alkaline phosphatase the dominant component and a very rapid decline in phosphatase activity below pH 5.0. For the Breton samples the optimum activity occurs in a broad peak over the range pH 6.0-8.0, with acid or "neutral" phosphatase apparently the dominant component (Figure 5). These curves represent the relationship between phosphatase activity and buffer pH for air-dry soil samples, and cannot be extended to soil samples in which moisture or nutrient conditions change the relative sizes of the various components of soil phosphatase activity. Curves for air-dry samples represent for the most part the activity-pH relationship of accumulated enzyme. Both soils are acid (Table 12). The samples of the Breton soil were obtained from plots which had been limed, as the pH values indicate.

2.3.2 Kinetic constants

Measurement of phosphatase activity of air-dry surface samples of the two soils at increasing concentrations of substrate shows saturation with substrate, a characteristic of enzyme-catalyzed reactions (Figures 6 and 7). For the Malmo soil samples the substrate concentrations used were not high enough to saturate the system (with substrate), and the curve only approaches the zone of zero order kinetics. The Hanes-Woolf transformation ($[S]/v_o$ versus $[S]$) of the data produces a

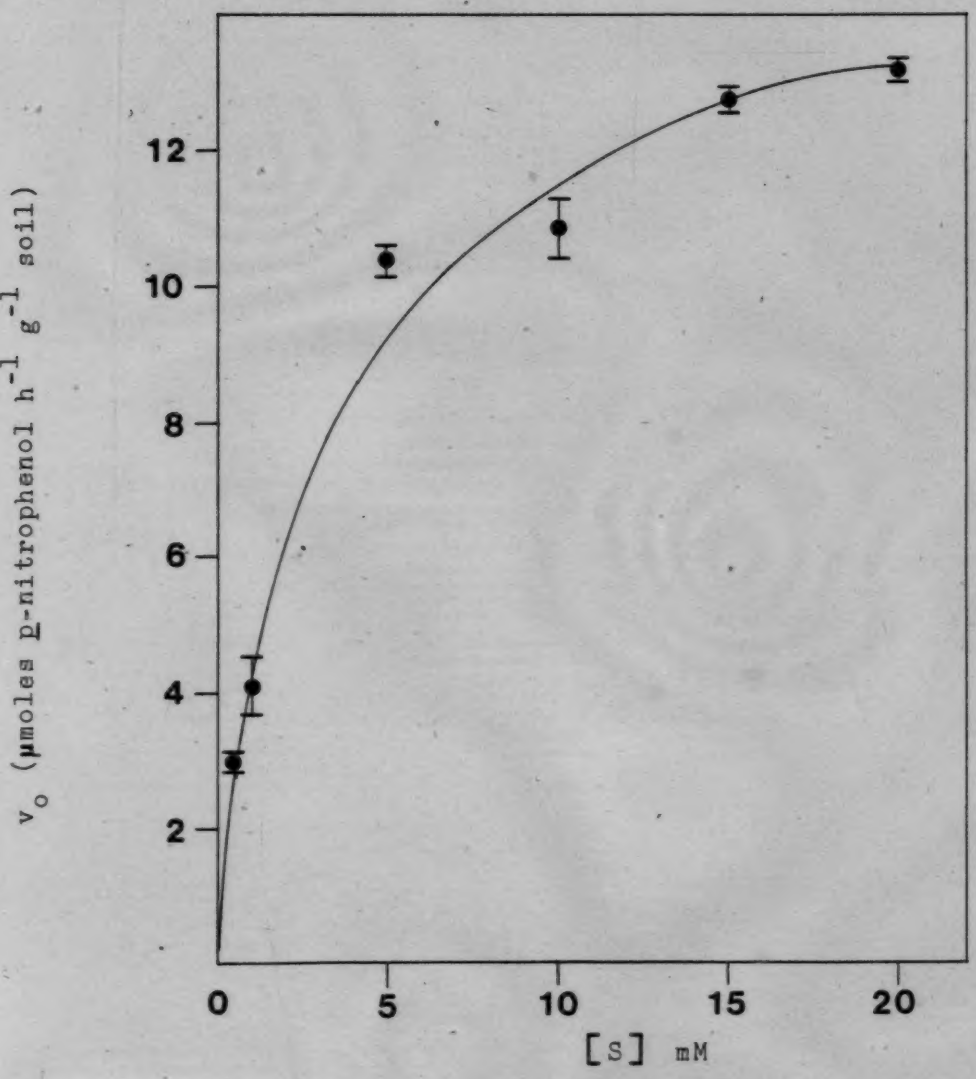


Figure 6. Plot of initial reaction velocity (v_o) versus initial substrate concentration ([S]) for alkaline (pH 8.0) phosphatase activity of air-dry samples of the Malmo Ap horizon; vertical bars represent one standard deviation.

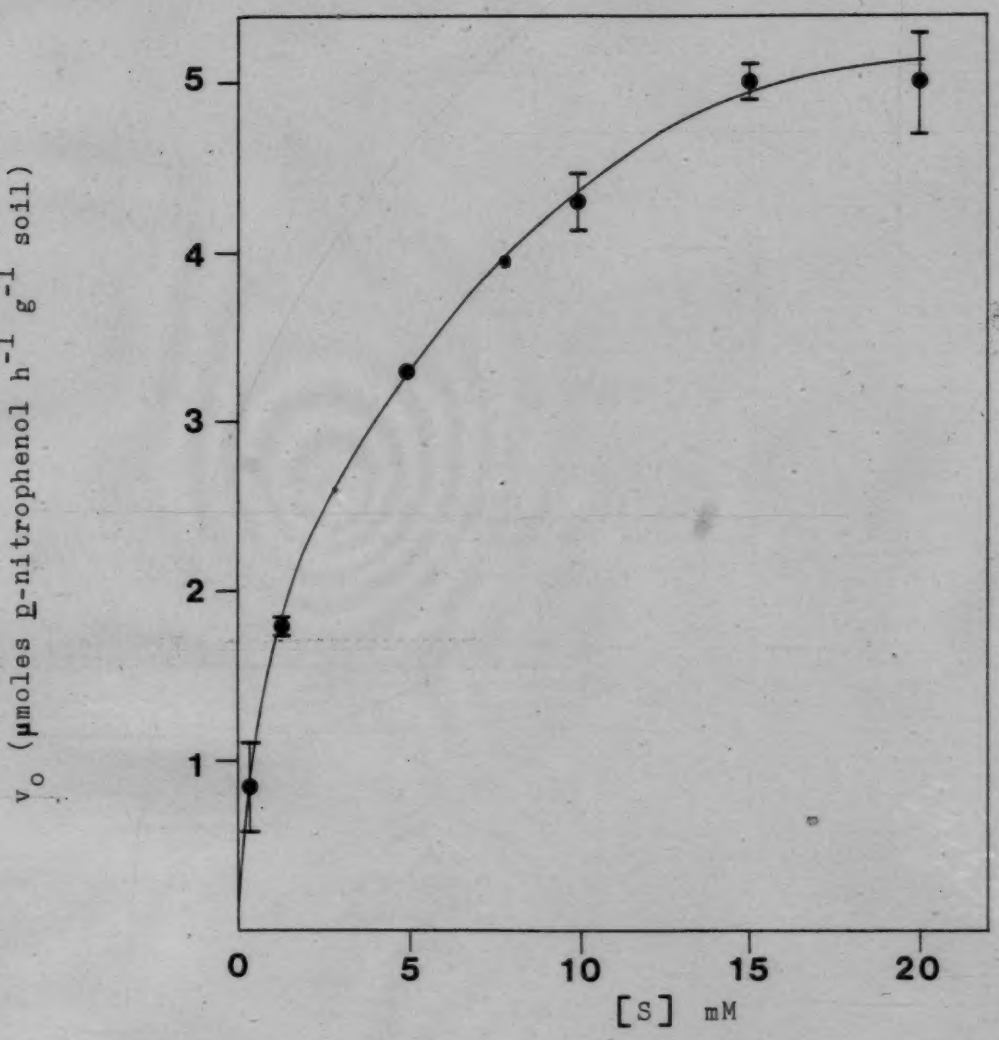


Figure 7. Plot of initial reaction velocity (v_o) versus initial substrate concentration ([S]) for acid (pH 6.0) phosphatase activity of air-dry samples of the Breton Ap horizon; vertical bars represent one standard deviation.

linear plot for both soils (Figures 8 and 9), with coefficients of determination (r^2 values) of 0.996 and 0.989 for the Malmo and Breton samples respectively. Simple linear regression of $[S]/v_o$ on $[S]$ yields K_m values of 2.3 and 2.2 mM, and V_{max} values of 14.5 and 5.6 µmoles p-nitrophenol h^{-1} g^{-1} soil for the Malmo and Breton samples respectively (Table 13). These K_m values are similar to those reported in the literature for soils (Table 9). The V_{max} values of samples of the Malmo and Breton soils are higher (especially for the Malmo soil) than values of V_{max} reported in the literature for soil phosphatase activity (Table 9). For the Breton samples, the calculated value of V_{max} (5.6 µmoles p-nitrophenol h^{-1} g^{-1} soil) is less than the value of 5.8 µmoles p-nitrophenol h^{-1} g^{-1} soil at pH 6.0 obtained in determination of the pH curve. This is most probably due to variation in the amounts of enzyme in the samples of the Breton soil used, together with the effect of storage.

The Eadie-Hofstee plots (v_o versus $v_o/[S]$) for both soils are nonlinear (Figures 10 and 11), suggesting that phosphatase activity does not follow Michaelis-Menten kinetics for solutions. Irving and Cosgrove (1976) found similar nonlinearity in Eadie-Hofstee plots of phosphatase activity measurements of a kraznozem.

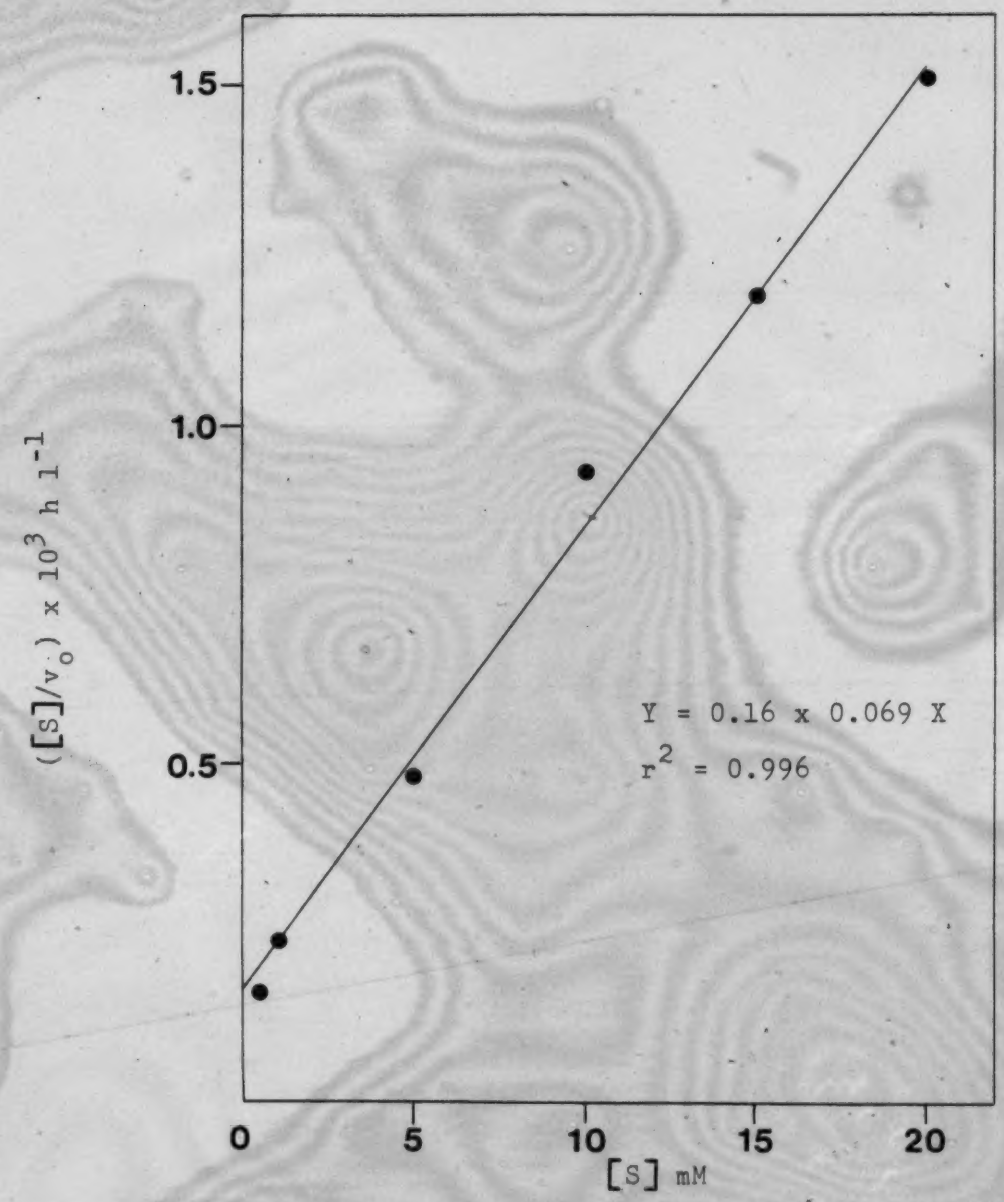


Figure 8. Hanes-Woolf plot of the ratio of initial substrate concentration to initial reaction velocity ([S]/v_o) versus the initial substrate concentration ([S]) for alkaline (pH 8.0) phosphatase activity of air-dry samples of the Malmo Ap horizon.

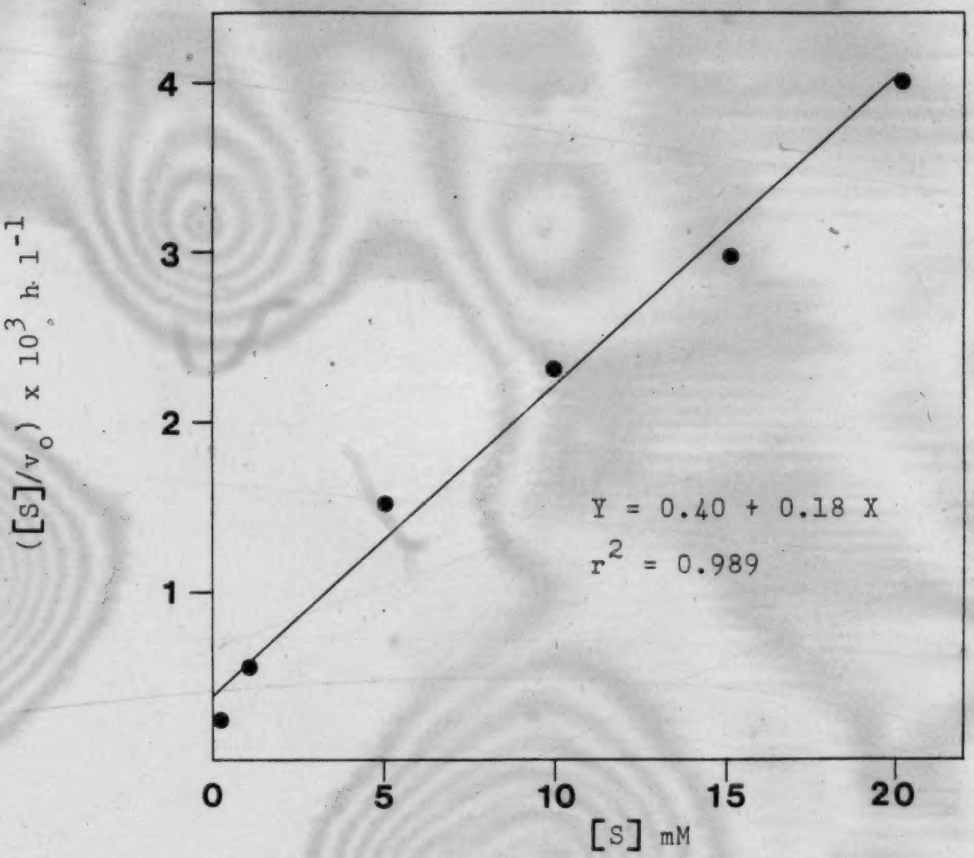


Figure 9. Hanes-Woolf plot of the ratio of initial substrate concentration to initial reaction velocity ([S]/v_o) versus the initial substrate concentration ([S]) for acid (pH 6.0) phosphatase activity of air-dry samples of the Breton Ap horizon.

Table 13. Values of K_m and V_{max} for air-dry samples of the Malmo and Breton Ap horizons.*

Soil samples	K_m (mM)	V_{max} (μmoles p-nitrophenol h^{-1} g^{-1} soil)
Malmo	2.3	14.5
Breton	2.2	5.6

* values of K_m and V_{max} obtained as described in the methods section and Appendix 2 by simple linear regression from Hanes-Woolf transformations of the data.

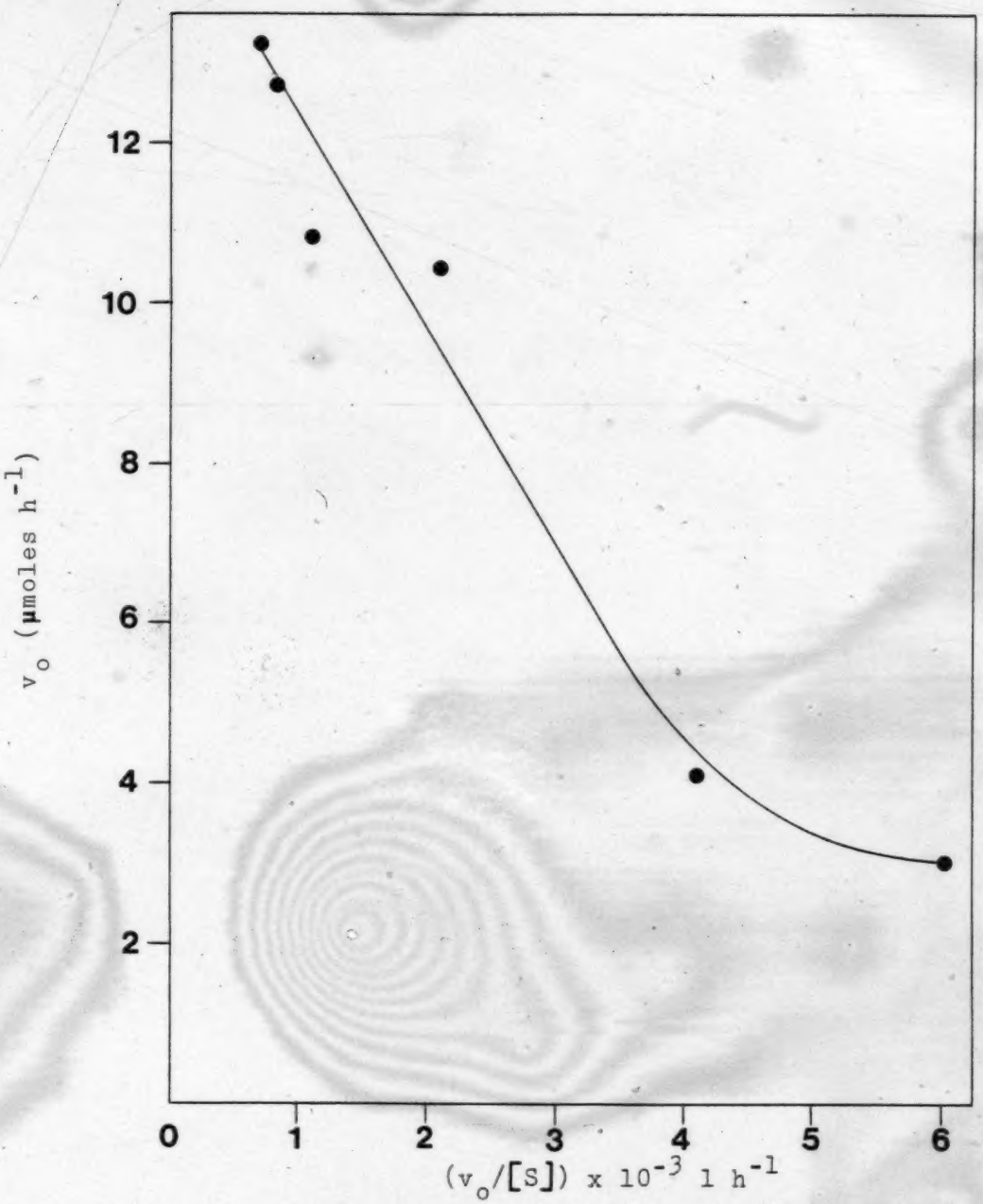


Figure 10. Eadie-Hofstee plot of the initial reaction velocity (v_o) versus the ratio of initial reaction velocity to initial substrate concentration ($v_o/[S]$) for alkaline (pH 8.0) phosphatase activity of air-dry samples of the Malmo Ap horizon.

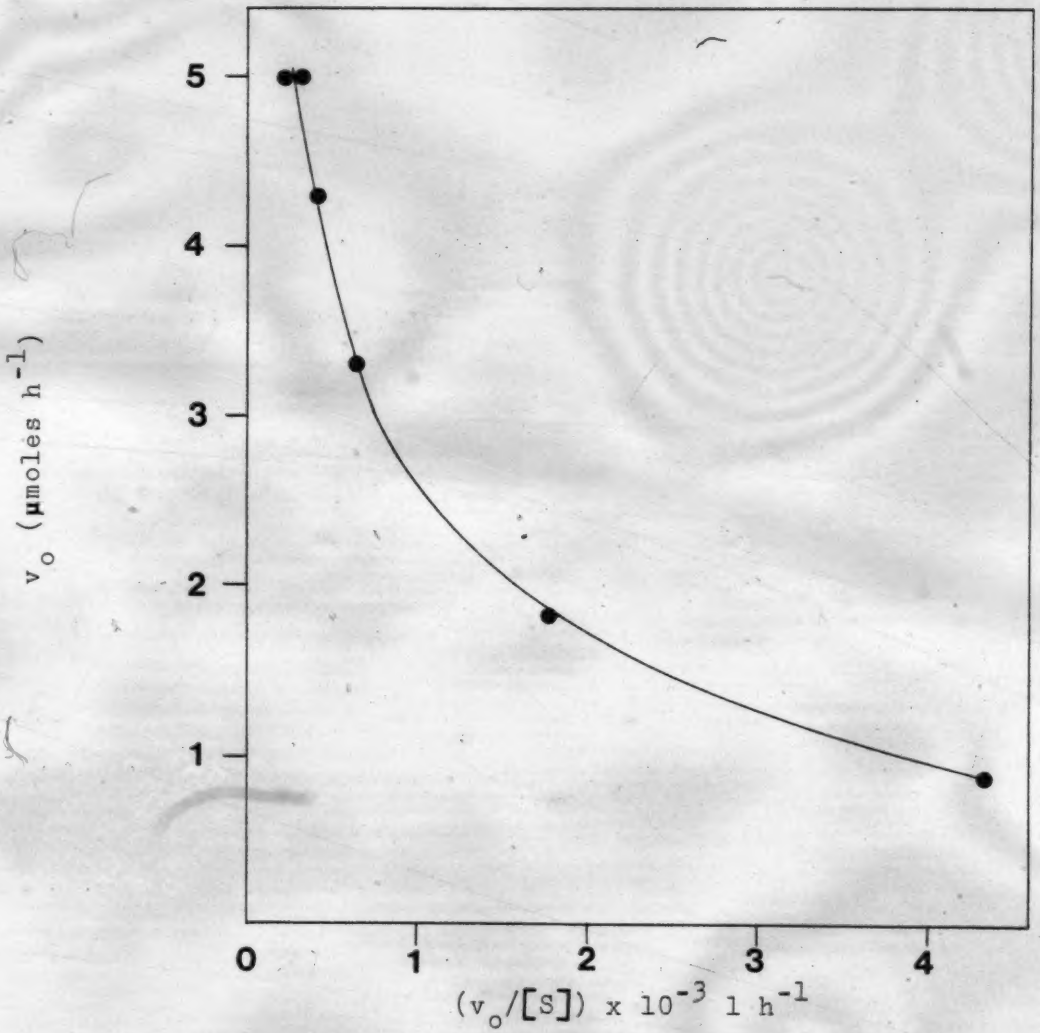


Figure 11. Eadie-Hofstee plot of the initial reaction velocity (v_o) versus the ratio of initial reaction velocity to initial substrate concentration ($v_o/[S]$) for acid (pH 6.0) phosphatase activity of air-dry samples of the Breton Ap horizon.

2.4 Discussion

Speir and Ross (1978) stated that assay of phosphatase activity with artificial substrates such as p-nitrophenyl phosphate often yields two pH optima, one acid (pH 4-6), and the other alkaline (pH 8-10); either optimum, or both, may be present. The results obtained for samples of the Malmo soil agree with this consensus, showing an alkaline optimum at pH 8.0-9.0. The activity-pH curve of the Breton soil samples resembles a pattern reported in other studies—a broad peak about a nearly neutral optimum—and represents a mixture of acid and alkaline phosphatase (Speir and Ross, 1978). Eivazi and Tabatabai (1977) and Juma and Tabatabai (1978) concluded from measurements of phosphatase activity in air-dry surface samples of soils with a wide range of physical and chemical properties that acid phosphatase is predominant in acid soils, and alkaline phosphatase is predominant in alkaline soils. This relationship does not always hold, as is evident for the Malmo soil samples where alkaline phosphatase is predominant in a soil with acid pH. Furthermore, the results of this study suggest that phosphatases in soil, whether associated with an actively growing microbial population or stabilized as accumulated enzymes, are not necessarily in an environment where pH conditions are optimal for activity.

The two components of soil phosphatase activity (acid and alkaline phosphatase) represent varied sources of enzyme. Gould et al. (1979) suggested that in soil acid phosphatase is primarily of plant origin, while alkaline phosphatase is primarily of microbial origin. Fungi may also be major contributors of acid phosphatase in soil (Casida, 1959). Determination of sources of phosphatases in soil on the basis of measured pH optima is complicated by stabilization of phosphatases, which may affect pH optima determined for activity of soil samples (Ramirez-Martinez and McLaren, 1966b). If the effect of stabilization on pH optima is discounted, the results of this study indicate a contribution of both plant and microbial phosphatase in the Breton soil, as opposed to a major contribution of microbial (alkaline) phosphatase in the Malmo soil. It is of interest to consider what causes the accumulation of alkaline phosphatase in the Malmo soil.

The nonlinearity of the Eadie-Hofstee plots, which is evidence of discrepancy in fit of Michaelis-Menten kinetics for solutions to phosphatase activity of soil samples, indicates that the K_m values obtained from the Hanes-Woolf plots are apparent K_m values for soil samples in a batch-type assay. These apparent K_m values reflect the proportions of the various forms of phosphatase present in the soil samples, and represent an average

measure of affinity of enzyme for substrate for all microsites.

The effect of microenvironment on activity of stabilized phosphatase of air-dry soil samples is indicated by the similarity of K_m values reported for different soils, differences in K_m values of soil samples and enzymes in solution, and discrepancy in fit of Michaelis-Menten kinetics to measurements of soil phosphatase activity. Similarity in K_m values of phosphatase activity of soils of different geographical locations (Table 9), and within this study between samples of two genetically different soils (a Gray Luvisolic soil and a Black Chernozemic soil), is consistent with the conclusion of Tabatabai and Bremner (1971) that phosphatases in different soils are either similar in type and origin, or the K_m values are made uniform in soil by association with soil constituents. Differences in pH optima reported for soil phosphatase activity and the wide range of soil organisms capable of producing phosphatase suggest that the influence of the microenvironment (as opposed to similarities in type and origin of enzymes) makes K_m values uniform, particularly in air-dry soil samples in which the predominant component of activity is accumulated enzyme.

The K_m values for the Malmo and Breton soil samples are similar in size to the larger values reported for plant and microbial phosphatases in solution (Table 14).

Table 14. Values of K_m for phosphatases from various plant and microbial sources.*

Enzyme and source	K_m (mM)
purified nonspecific acid (pH 5.3) phosphatase of Lupinus albus (Newmark and Wenger, 1960)	3.2×10^{-1}
root acid (pH 5.5) phosphatase of Bouteloua gracilis (Gould et al., 1979)	3.46×10^{-1}
purified acid (pH 5.6) phosphatase of potato (Neumann, 1968)	2.5×10^{-1}
purified acid (pH 6.5) phosphatase of potato (Tabatabai and Bremner, 1971)	32.8
purified acid (pH 6.5) phosphatase of wheat germ (Tabatabai and Bremner, 1971)	6.81
purified acid (pH 5.6) phosphatase of Neurospora crassa (Kuo and Blumenthal, 1961)	2.5
purified alkaline (pH 9.0) phosphatase of Escherichia coli (Neumann, 1968)	9.4×10^{-2}
acid (pH 3.65) and alkaline (pH 8.9) phosphatase of Saccharomyces cerevisiae (Schurr and Yagil, 1971)	4.5×10^{-1}

* with p-nitrophenyl phosphate as substrate

The similarity is surprising because K_m values of air-dry soil samples would be expected to be higher than those for plant and microbial phosphatases in solution, reflecting microenvironmental effects which restrict the access of substrate to enzyme. Such is the case for smaller values reported in Table 14, and the values of Francis and King (1979) in Table 15, which differ from the soil K_m values obtained in the present study by 1-3 orders of magnitude. Differences in assay methods and purification of enzyme preparations prevent strict comparison of K_m values. Higher K_m values of phosphatase activity of air-dry soil samples result from adsorption of enzyme and substrate by clays (Cervelli et al., 1973; Makboul and Ottow, 1979), and formation of carbohydrate-enzyme and humocarbohydrate-enzyme complexes (Batistic et al., 1980), as well as other factors.

Nonlinearity of the Eadie-Hofstee plots for samples of both soils is in agreement with results obtained by Irving and Cosgrove (1976), and supports the hypothesis that activity of phosphatases in soil is modified by microenvironmental factors which are not considered in Michaelis-Menten kinetics for solutions. At the microsite Michaelis-Menten kinetics describe only part of the enzyme action, and other mechanisms must be added to model effects such as substrate adsorption and dif-

Table 15. Values of K_m for alkaline phosphatase of Escherichia coli by two methods (from Francis and King, 1979).

	Method	pH	K_m (mM)
1.	Measurement of intact cell alkaline phosphatase activity with β-glycerophosphate as substrate.	6.0	1.01×10^{-2}
		5.6	1.31×10^{-2}
		5.2	2.50×10^{-2}
2.	An in vivo assay of alkaline phosphatase at suboptimal (acidic) pH with very low concentrations of substrate (β-glycerophosphate) through kinetic analysis of P_i uptake data.	5.6	3.1×10^{-3}
		5.2	6.0×10^{-3}

fusion, as Irving and Cosgrove (1976) have proposed.

2.5 Conclusions

The results of this study support the following conclusions :

(i) the different activity-pH curves for the two soils indicate differences in the relative amounts of plant and microbial phosphatase present, and indicate a predominance of microbial (alkaline) phosphatase in the Malmo soil.

(ii) the data suggest a multiple enzyme-environment system, even in dried soils which are dominated by accumulated enzyme.

(iii) the similarity in K_m values for phosphatase activity of air-dry samples of the two soils supports the hypothesis that microenvironment influences the activity of accumulated phosphatase.

(iv) differences in K_m values for samples of the two soils and those of plant and microbial phosphatases in solution may result from the effect of microenvironment in reducing accessibility of substrate to enzyme.

Data to Accompany

Figures 4 and 5

Table 1. Relationship between phosphatase activity and pH for air-dry samples of the surface horizon of the Malmo SiCL.

Buffer pH	Phosphatase activity (μmoles p-nitrophenol h^{-1} g^{-1} soil) $\bar{X} \pm s$
4.0	0.39 ± 0.025
4.5	6.7 ± 0.25
5.0	11.2 ± 0.26
6.0	11.6 ± 0.57
7.0	11.9 ± 0.50
8.0	14.2 ± 0.25
8.5	14.1 ± 0.77
9.0	13.5 ± 0.47
10.0	12.8 ± 0.32

Table 2. Relationship between phosphatase activity and pH for air-dry samples of the surface horizon of the Breton L-SiL.

Buffer pH	Phosphatase activity (μmoles p-nitrophenol h^{-1} g^{-1} soil) $\bar{X} \pm s$
4.0	3.5 ± 0.24
5.0	4.5 ± 0.44
6.0	5.8 ± 0.43
6.5	5.6 ± 0.39
7.0	5.6 ± 0.25
8.0	5.4 ± 0.32
9.0	4.3 ± 0.30
10.0	3.7 ± 0.15

Calculation of K_m and V_{max} for the Malmo and Breton Soils

Table 1. Values calculated from the data for the Hanes-Woolf transformation of the Michaelis-Menten equation.*

Soil**	[S] (mM)	v_o (μmoles h^{-1})	[S]/v_o (x 10^3 h l^{-1})
Malmo	0.5	3.0	0.17
	1	4.1	0.24
	5	10.4	0.48
	10	10.8	0.93
	15	12.7	1.18
	20	13.2	1.51
Breton	0.2	0.86	0.23
	1	1.8	0.55
	5	3.3	1.51
	10	4.3	2.33
	15	5.0	3.00
	20	5.0	4.00

* $$\frac{[S]}{v_o} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot [S]$$

** 1.0 g oven-dry soil

Calculation for the Malmo soil

Letting X and Y represent [S] and $[S]/v_o$, respectively;

$\bar{X} = 8.58$ $\qquad$ $\bar{Y} = 0.75$

$$\Sigma X^2 - \frac{(\Sigma X)^2}{n} = 309 = A \qquad \Sigma Y^2 - \frac{(\Sigma Y)^2}{n} = 1.46 = B$$

$$\Sigma XY - \frac{\Sigma X \, \Sigma Y}{n} = 21.2 = C$$

$$b_{yx} = \frac{\Sigma XY - \frac{\Sigma X \, \Sigma Y}{n}}{\Sigma X^2 - \frac{(\Sigma X)^2}{n}} = 0.069$$

$$a = \bar{Y} - b\bar{X}$$
$$= 0.75 - 0.069(8.58)$$
$$= 0.16$$

$$Y = a + bX$$
$$= 0.16 + 0.069\,X$$

$$r_{xy} = \frac{C}{(A \times B)^{\frac{1}{2}}} = 0.998^{**} \qquad r^2 = 0.996$$

$1/V_{max} = b = 0.069$ h μmoles^{-1}

$V_{max} = 1/0.069$ h μmoles^{-1} = 14.5 μmoles h^{-1} g^{-1} soil

$K_m/V_{max} = 0.16 \times 10^3$ h l^{-1}

$K_m = V_{max} \times 0.16 \times 10^3$ h l^{-1}

= 14.5 μmoles h^{-1} x 0.16×10^3 h l^{-1}

= 2.3 mM

Calculation for the Breton soil

Letting X and Y represent [S] and [S] /v_o, respectively;

$\bar{X} = 8.53$ $\bar{Y} = 1.94$

$\Sigma X^2 - \frac{(\Sigma X)^2}{n} = 314 = A$ $\Sigma Y^2 - \frac{(\Sigma Y)^2}{n} = 10.6 = B$

$\Sigma XY = \frac{\Sigma X \Sigma Y}{n} = 57.3 = C$

$$b_{yx} = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{n}}{\Sigma X^2 - \frac{(\Sigma X)^2}{n}} = 0.18$$

$a = \bar{Y} - b\bar{X}$

= 1.94 - 0.18(8.53)

= 0.40

$$Y = a + bX$$

$$= 0.40 + 0.18\ X$$

$$r_{xy} = \frac{C}{(A \times B)^{\frac{1}{2}}} = 0.995^{**} \qquad r^2 = 0.989$$

$$1/V_{max} = b = 0.18\ h\ \mu moles^{-1}$$

$$V_{max} = 1/0.18\ h\ \mu moles^{-1} = 5.6\ \mu moles\ h^{-1}\ g^{-1}\ soil$$

$$K_m/V_{max} = 0.40 \times 10^3\ h\ l^{-1}$$

$$K_m = V_{max} \times 0.40 \times 10^3\ h\ l^{-1}$$

$$= 5.6\ \mu moles\ h^{-1} \times 0.40 \times 10^3\ h\ l^{-1}$$

$$= 2.2\ mM$$

CHAPTER 3
CONTROL OF PHOSPHATASE ACTIVITY BY ORTHOPHOSPHATE IN INCUBATED SOIL SAMPLES

3.1 Introduction

3.1.1 Effectiveness of extraction methods in measuring soil labile P

Phosphatase activity measurements indicate levels of phosphatase in soil and potential activity, but do not represent activity *in situ*. Therefore, mineralization of organic P compounds in soil must be assessed by some other measurement. Because of difficulties in measuring concentrations of inorganic and organic P in the soil solution, measurements of labile P by extraction methods have been used to follow mineralization of a labile organic P fraction in soil (Halm *et al.*, 1972; Bowman and Cole, 1978; Cole *et al.*, 1978; Dick and Tabatabai, 1978).

Abbott (1978) reported that in 0.5 M $NaHCO_3$ extracts of samples of a calcareous soil there was a high correlation between inorganic P and uptake of P by the test crop, and correlation coefficients for total P in the extracts were lower than for inorganic P. He suggested that either a large portion of the organic P in the $NaHCO_3$ extracts

was resistant to natural conversion to inorganic P, or the inorganic P in the extracts may include soil organic P hydrolyzed during the 30-minute extraction period. In regard to the latter hypothesis, Dick and Tabatabai (1977) reported interference from hydrolysis of acid-labile organic P compounds during orthophosphate measurements by the method of Murphy and Riley (1962).

Bowman and Cole (1978) tested the hypothesis that in 0.5 M $NaHCO_3$ extracts of soil samples the organic P component measures a labile or quickly mineralized organic P fraction while the inorganic P component reflects mineralization of the extractable organic P fraction. Various unlabelled organic P compounds were added to samples of a sandy loam prior to incubation at field capacity. After 3 days, glycerophosphate and four 3' nucleotides of ribonucleic acid (RNA) were completely mineralized and accounted for as inorganic P in the $NaHCO_3$ extract or as P immobilized in microbial cells (from the $NaHCO_3$-extractable inorganic P pool). While RNA degraded in 18 days, sodium-inositol hexaphosphate was unrecoverable in the $NaHCO_3$ extract upon immediate extraction, and was relatively unaffected over the 18-day incubation period, indicating that this substrate was precipitated or adsorbed in the soil samples. The $NaHCO_3$ extract did not seem to extract live microbial cells; thus the decrease in extractable inorganic P from immobilization was not reflected in increases in the extractable organic P

fraction. Because a relatively constant amount of native organic P was extracted from the soil samples, changes in the level of organic P in the $NaHCO_3$ extract during incubation represented changes in the size of a labile organic P pool.

Dick and Tabatabai (1978) measured rates of hydrolysis of seven organic P compounds in soil samples incubated under aerobic and waterlogged conditions by determination of orthophosphate extracted by 0.5 M H_2SO_4. The compounds were added to the soil samples at a rate of 500 ppm P, a concentration designed to approximate that near a fertilizer band. Monoesters (monomethyl phosphate, ß-glycerophosphate, glucose-1-phosphate, phenyl phosphate, and p-nitrophenyl phosphate) were completely hydrolyzed in samples of two acid soils after seven days of incubation. Diesters (diphenyl phosphate and bis-p-nitrophenyl phosphate) were hydrolyzed at slower rates.

The experimental results cited above indicate that extractable inorganic P measurements can be used to follow mineralization of readily hydrolyzed organic P compounds added to soil. Further, the organic P component of $NaHCO_3$ extracts represents a labile, quickly mineralized organic P pool together with an organic P fraction which is not readily hydrolyzable.

Investigations of the nature of immobilized P in amended soil samples indicate that much of the mobile organic P fraction of the soil solution or soil extracts

is contained within cellular debris, and is relatively stable against hydrolysis by extracellular phosphatases. For example, Hannapel et al. (1964a, b) examined the mobile P fraction of soil solutions displaced from columns of amended samples of a calcareous sandy loam. More than 95% of the P moving was organic, and millipore filtration showed it to be contained within microbial cells and cellular debris. Martin (1970) examined cold and hot water extracts of samples of ten soils incubated with water containing ^{32}P-orthophosphate under conditions favoring microbial activity. Although hot extracts contained more than 30 ^{32}P-labelled low molecular weight phosphate esters of bacterial origin, most of the organic P of water extracts of the soil samples appeared to be very stable, with a low turnover rate.

3.1.2 Significance of labile P in the field

Variation in levels of labile P in the field has been related to the cycling of P in the plant-soil system. Halm et al. (1972) observed large seasonal fluctuations in levels of 0.5 M $NaHCO_3$-extractable organic P in a Brown Chernozemic soil under native grassland. A low available P status at the site together with a lack of plant response to application of fertilizer P suggested that mineralization of labile organic P balanced uptake of P by new plant material. An increase in $NaHCO_3$-extractable P coincided with the period of maximum plant growth,

highest microbial activity, and lowest level of P in litter.

Abbott (1978) measured inorganic and organic P in five extracts of soil samples collected during growth of sorghum in the field. Organic P in four extracts (0.5 M $NaHCO_3$, 0.02 N HCl, 0.02 N NaCl, and water aided by gelatin and Superfloc) increased in midsummer during a period when microbial growth was stimulated by small grain residue and high soil temperatures. He concluded that estimation of available P is improved by including measurements of extractable organic P when a significant reserve of available P exists in organic form as a result of immobilization.

Stewart et al.(1980) fractionated P in soil samples from plots under permanent pasture and from a wheat-fallow rotation in Saskatchewan. Approximately 40% of the P lost during cultivation in the wheat-fallow rotation was associated with the more labile inorganic and organic components extracted by an anion exchange resin, 0.5 M $NaHCO_3$, and 0.1 M NaOH.

These three investigations of variation in levels of labile P in the field suggest that turnover of labile organic P is involved in supply of P to plants. Further, extractable organic P measurements can be used to follow temporal changes in the size of the labile organic P pool in the field. The results of Halm et al. (1972) and Abbott

(1978) also suggest that the labile organic P fraction may be partly made up of organic P from microbial cells.

3.1.3 Efficiency of orthophosphate supply from the labile P fraction in incubated soil samples

Soil incubation studies have provided information about the efficiency of orthophosphate supply from the labile P pool to a proliferating microbial population. Chauhan et al. (1979) investigated movement of P between soil inorganic, organic, and biomass P compartments in incubated samples of the Ap horizon of a Black Chernozemic soil which received dried grass or cellulose plus NH_4NO_3 with and without KH_2PO_4 every 30 days. For the cellulose plus NH_4NO_3 treatment, the microbial P pool increased rapidly at the beginning of each incubation period, representing rapid immobilization of P from a pool recharged by either desorption and dissolution of inorganic P, or mineralization of organic P. The $NaHCO_3$-extractable P fraction was less dynamic than was biomass P. Extractable inorganic P decreased during the initial buildup of the microbial population. Although changes in extractable organic P were less consistent, this fraction tended to decrease slightly during the periods of increase in microbial P. These results confirmed the need for refinement of methods for defining labile P on a kinetic basis, because the $NaHCO_3$-extractable P measurements failed to reflect the dynamics of P transformations.

In another experiment, Chauhan et al. (1981) added cellulose plus NH_4NO_3 with and without KH_2PO_4 every 30 days to incubated samples of two soils, one with low and the other with high labile inorganic P status (3 and 18 ug $NaHCO_3$-extractable P g^{-1} soil respectively). Added KH_2PO_4 increased CO_2 production in both soils. In the soil with low labile P status, after the first addition of cellulose plus NH_4NO_3 the supply of labile inorganic P dropped to a minimum level (less than 1 ug $NaHCO_3$-extractable P g^{-1} soil) where it remained for the duration of the incubation period. In spite of the low level of labile inorganic P, microbial P consistently increased over the first four days of incubation following addition of cellulose and NH_4NO_3. Furthermore, at the end of the incubation period the amount of cellulose-C remaining was only 5% higher in the treatment without added P (relative to the treatment with added P). The authors concluded that increased mineralization of labile organic P partially compensated for lack of P in the cellulose and NH_4NO_3 treatment.

Stewart et al. (1980) fractionated P in samples of two soils following amendment with cellulose plus NH_4NO_3 with and without KH_2PO_4 at 30-day intervals over a 270-day incubation period. In the soil with high labile inorganic P status, there was a buildup of 0.1 M NaOH-extractable organic P. In the soil with low labile inorganic P status, with the cellulose plus NH_4NO_3 treat-

ment there was not a similar buildup of 0.1 M NaOH-extractable organic P. The authors postulated that mineralization of microbial metabolites prevented accumulation of 0.1 M NaOH-extractable organic P in the soil with low labile inorganic P status, in the absence of added P, a conclusion consistent with the hypothesis of McGill and Cole (1981).

The experimental results cited above provide indirect evidence in support of the hypothesis that mineralization of labile organic P is involved in supply of P to the proliferating microbial population in amended soil samples. Nevertheless, P supply by rapid desorption and dissolution of inorganic P cannot be discounted.

3.1.4 Conclusions and experimental objectives

The literature reviewed indicates that:

(i) incubation studies have demonstrated a repressible-derepressible synthesis of acid phosphatase by the proliferating microbial population of amended soil samples. The effect of orthophosphate on phosphatase activity is due more to its effect on enzyme synthesis than on activity of existing enzyme.

(ii) in addition to control of enzyme synthesis and activity by orthophosphate, activity of phosphatase <u>in situ</u> is affected by availability of substrate.

(iii) measurement of labile P in incubated soil samples and samples obtained in the field suggests

that mineralization of labile organic P is involved in supply of P to microorganisms and plants in the absence of sufficient labile inorganic P. The evidence, however, is not conclusive. (iv) measurement of labile P by extraction methods is an inadequate means of looking at the P mineralization-immobilization balance.

The objectives of the present investigation were to test further the hypothesis that at the microsite in soil the microbial population exhibits a response to the orthophosphate concentration of the soil solution which is similar to that demonstrated for various microorganisms grown in solution culture. Whereas previous studies examined control of soil phosphatase activity by orthophosphate in a single incubation period, the present study was designed to examine control by orthophosphate over two successive incubation periods, each following a pulse of nutrients into the soil samples. Literature reviewed suggests that phosphatase measurements may represent real activity associated with mineralization of naturally occurring substrate in situ. This hypothesis was tested by examining the production and persistence of phosphatase in incubated soil samples, together with concomitant changes in labile P and in CO_2 evolution.

3.2 Materials and Methods

3.2.1 Soils

The soil samples used in this study were obtained from the Ap horizon of the Breton L-SiL. The samples were collected in October of 1977 from the Breton Plots of the Department of Soil Science, University of Alberta (NE-25-47-4 W5), Series D Plot No. 5 (under hay, with no fertilizer applied), and air-dried. Before use in this study the soil samples were ground and sieved (less than 2 mm), then preincubated for approximately 10 months (300 days) at field capacity and 22-25 C in the dark. Preincubation of the samples established a relatively stable microbial population respiring at a low level.

3.2.2 Experimental design

This laboratory study extended over two successive incubation periods, each following amendment of the soil samples. The treatments were selected to provide comparisons among a control (moistened soil), and soil with a microbial population growing under two sets of conditions (with respect to phosphatase synthesis)—conditions of derepression followed by repression on one hand, and conditions of repression followed by derepression on the other. Samples of preincubated soil (150 g, oven-dry weight) were amended in two pulses of nutrients, one at the beginning of each incubation period, with treatments as follows:

(i) moistened soil (control)

(ii) soil receiving C (5 mg glucose-C g^{-1} soil) plus N (438 µg NH_4NO_3-N g^{-1} soil) in the first pulse, and C plus N (as above) plus P (200 µg KH_2PO_4-P g^{-1} soil) in the second pulse.

(iii) soil receiving C plus N plus P (as above) in the first pulse, and C plus N (as above) in the second pulse.

The control treatment was replicated four times, while the other two treatments were replicated six times.

For each replicate of treatments 2 and 3, the soil sample was spread on wax paper and the nutrients added—glucose was sprinkled onto the surface of the soil, while NH_4NO_3 and KH_2PO_4 were dissolved in 5 ml of deionized water and added dropwise. The soil sample was mixed, then returned to the container. Moisture content of all soil samples was adjusted to a level near field capacity. The samples were equilibrated at 4 C for 12 hours before incubation at 22-25 C in the dark. The moisture content of the soil samples was adjusted every second or third day by weight. At intervals the soil was sampled for measurement of respiration rate, labile P, and phosphatase activity.

The second incubation period (day 0) began on day 85 of the first period, with amendment of remaining soil as described above for the second pulse of nutrients.

3.2.3 Soil respiration measurements

All soil respiration measurements were obtained by a common procedure. On day 0 of each incubation period a subsample of moist soil (60 g in the first period and 40 g in the second) was removed from the incubation containers and placed into sealer jars of approximately 1 liter capacity. Thereafter, the soil in the jars was maintained at a level near field capacity by frequent additions of deionized water to bring the weight to the appropriate value. The jars were opened regularly between measurements for aeration. At selected times over the course of incubation aliquots (20 or 25 ml) of standard NaOH solution (0.200-1.00 M, depending upon the expected amount of CO_2 evolved) contained in 100-ml beakers were placed into the jars, which were subsequently sealed, to collect evolved CO_2. At the end of specified time intervals (usually 24 hours) the beakers were removed, saturated $BaCl_2$ solution and phenolphthalein indicator were added, and the residual NaOH titrated with standard 1.00 M HCl solution. The respiration rates were calculated from values obtained for the three treatments and blanks, after correction for controls.

The method used in this study for respiration measurements has two limitations. First, the values obtained represent mean respiration rates over measured time intervals. Second, respiration rates were measured for soil samples which were incubated in separate containers but

side by side with those samples used for the other analyses.

Results obtained were expressed as μg CO_2-C h^{-1} g^{-1} soil (oven-dry basis).

3.2.4 Labile P measurements

Labile P measurements were obtained with two extraction methods—Olsen's $NaHCO_3$ extraction and the modified medium-strength Bray method (McKeague, 1978, pp. 167-174), with some changes. In Olsen's method 4.9 g of moist soil were shaken with 80 ml of 0.5 M $NaHCO_3$ solution (pH 8.5) for 30 minutes, and then filtered under vacuum with Whatman No. 42 filter paper in Buchner funnels. In the modified Bray method 6.1 g of moist soil were shaken with 25 ml of 0.03 N NH_4F-H_2SO_4 solution and 2 scoops of Darco G60 activated carbon (checked for absence of P) for 2 minutes, then filtered through Whatman No. 40 filter paper. Both extracts were frozen directly after filtration to await analysis (Klingaman and Nelson, 1976).

Inorganic P in the thawed $NaHCO_3$ extracts was determined by the method of Watanabe and Olsen (1965), with measurement of the molybdenum blue color at 730 nm and correction of absorbance measurements for color resulting from organic matter in the extracts. The vanadomolybdophosphoric yellow color method (Jackson, 1958, pp. 151-154) was used to determine inorganic P in the thawed NH_4F-H_2SO_4 extracts, with absorbance measurements at 400 nm

and correction for P extracted from the activated carbon. In determination of inorganic P in the extracts, absorbance measurements were made at standard time intervals as soon as possible after stable color development to reduce the effect of hydrolysis of organic P esters in the extracts. Nevertheless, hydrolysis of organic P esters during measurement cannot be completely discounted (Dick and Tabatabai, 1977). Organic P was determined in the thawed $NaHCO_3$ extracts as the difference between total and inorganic P in the same extract solution. Total P was determined by a persulfate digestion method (Rand et al., 1976, p. 476), with modifications as indicated on pages 152-5, and measurement of inorganic P in the digest by the ascorbic acid method of Watanabe and Olsen (1965).

Labile P measurements (total, organic, and inorganic) were expressed as ug P g^{-1} soil (oven-dry basis).

3.2.5 Phosphatase activity measurements

Acid (pH 6.0) and alkaline (pH 8.0) phosphatase activity was assayed using the method of Tabatabai and Bremner (1969) with modifications introduced to eliminate the use of toluene, improve filtration, and speed the determination. Toluene has been used as a microbial inhibitor in assays of soil enzymes; it stops synthesis of enzymes by viable cells and prevents biological assimilation of the products of the enzymatic reactions. Use of

toluene has several disadvantages: toluene may affect cell permeability, unmask specific enzymes, and affect soil structure (Skujins, 1967; Speir and Ross, 1978). Various microorganisms isolated from soil decompose toluene (Skujins, 1967). For example, Kaplan and Hartenstein (1979) found that a variety of soil microorganisms (6 of 7 fungi imperfecti, 7 of 13 basidiomycetes, and 6 of 14 bacteria tested) were able to grow with 0.1 or 0.05% toluene as the sole source of carbon. Hence they concluded that use of toluene as a biostatic agent in measurements of extracellular enzyme activity may be invalid. Speir and Ross (1978) suggested that for short incubation periods (less than one hour) "sterilization" is not required where phosphatase activity is identical in toluene-treated and untreated samples. Such was the case for previously air-dry samples of the Malmo Ap horizon, a soil with a high level of phosphatase activity (Figure 12). Furthermore, in earlier work toluene was associated with cloudiness of the filtrate obtained in the assay procedure of Tabatabai and Bremner (1969). In light of the above, use of toluene was omitted in the revised assay procedure, and the incubation time reduced from 1 hour to 30 minutes. A large derepressed microbial population in incubated soil can produce a high level of phosphatase activity; for example, Spiers and McGill (1979) observed that in samples of Malmo soil amended with glucose and NH_4NO_3 the acid phosphatase activity reached

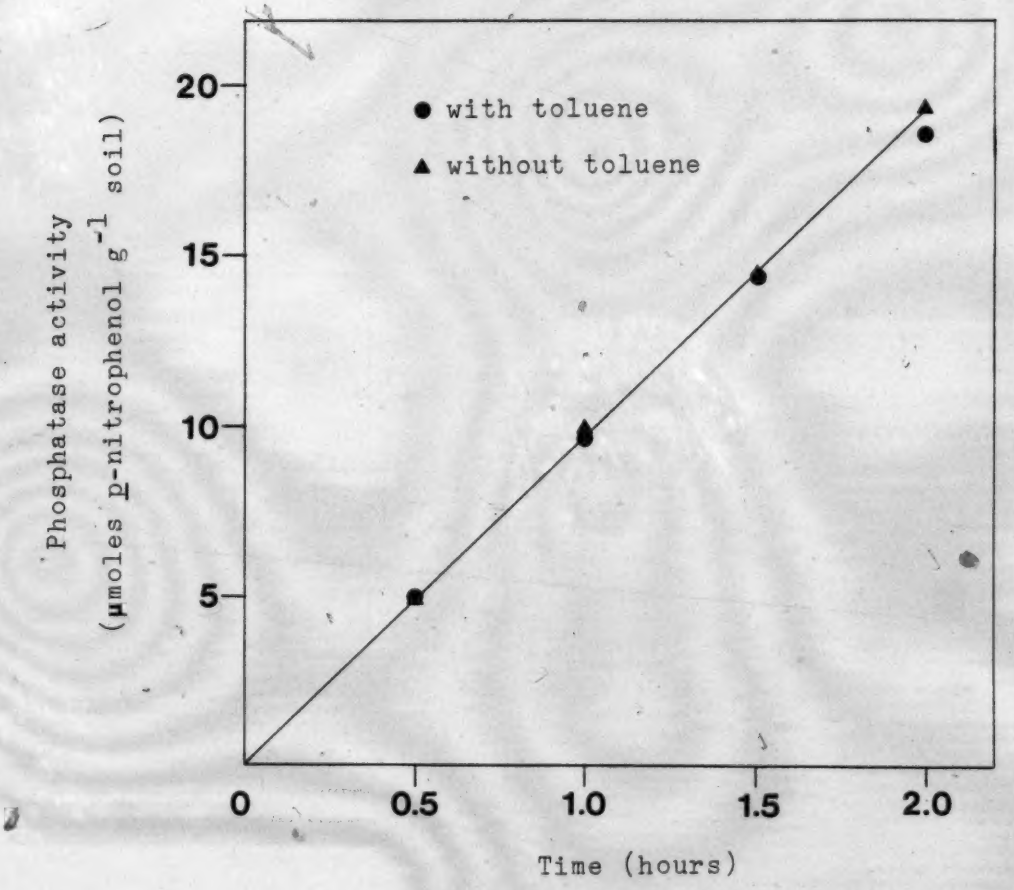


Figure 12. Effect of incubation time on alkaline phosphatase activity (with and without toluene) of previously air-dried samples of the Malmo Ap horizon. Each point plotted is the average of three replicates.

levels above 80 µmoles p-nitrophenol h^{-1} g^{-1} soil. The shorter incubation period reduces the effect of the high phosphatase activity in such a sample on the substrate concentration during the assay. A centrifugation step was introduced to improve separation of particulates from the filtrate.

In the revised procedure, 1.25 g of moist soil were incubated with 4 ml of Modified Universal Buffer (pH 6.0 for acid phosphatase and pH 8.0 for alkaline phosphatase) and 1 ml of substrate solution (0.075 M sodium p-nitrophenyl phosphate in appropriate buffer) in capped 50-ml plastic centrifuge tubes at 37 C for 30 minutes with shaking at 100 oscillations per minute. At the end of this period the reaction was stopped by addition of 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH, and the tubes were centrifuged at 740 Xg for 10 minutes, and the contents then filtered. The amount of p-nitrophenol released by soil phosphatase activity was determined colorimetrically (at 400 nm) in diluted aliquots of filtrate by comparison with a standard curve.

Results obtained were expressed as µmoles p-nitrophenol h^{-1} g^{-1} soil (oven-dry basis).

3.3 Results

3.3.1 First incubation period

In the first incubation period treatment 2 (glucose and NH_4NO_3) derepressed synthesis of both acid and alkaline phosphatase by the proliferating microbial population, while in treatment 3 (glucose, NH_4NO_3, and KH_2PO_4) phosphatase synthesis was repressed relative to treatment 2 (Figures 13, 14, and 15). Even with added KH_2PO_4 in treatment 3 there was some microbial production of phosphatase, which may represent synthesis of enzyme in response to a limited rate of recharge of solution orthophosphate levels in the immediate vicinity of the rapidly growing microbial population, together with possible incomplete mixing of added nutrients into the soil. For the Breton soil, the acid phosphatase component was more responsive to derepressing conditions than the alkaline component (Figure 13). The respiration data (Figures 16 and 17) show the flush of microbial growth expected from the addition of a readily oxidizable substrate such as glucose to soil. Lack of P in treatment 2 slowed respiration relative to that in treatment 3. This observation further suggests that the increase in phosphatase activity in treatment 2 is a response to an insufficient supply of soluble orthophosphate. A comparison of phosphatase and respiration data indicates that while the respiration rate for the two amended treatments returned to a level near that of the control in about 15 days, the

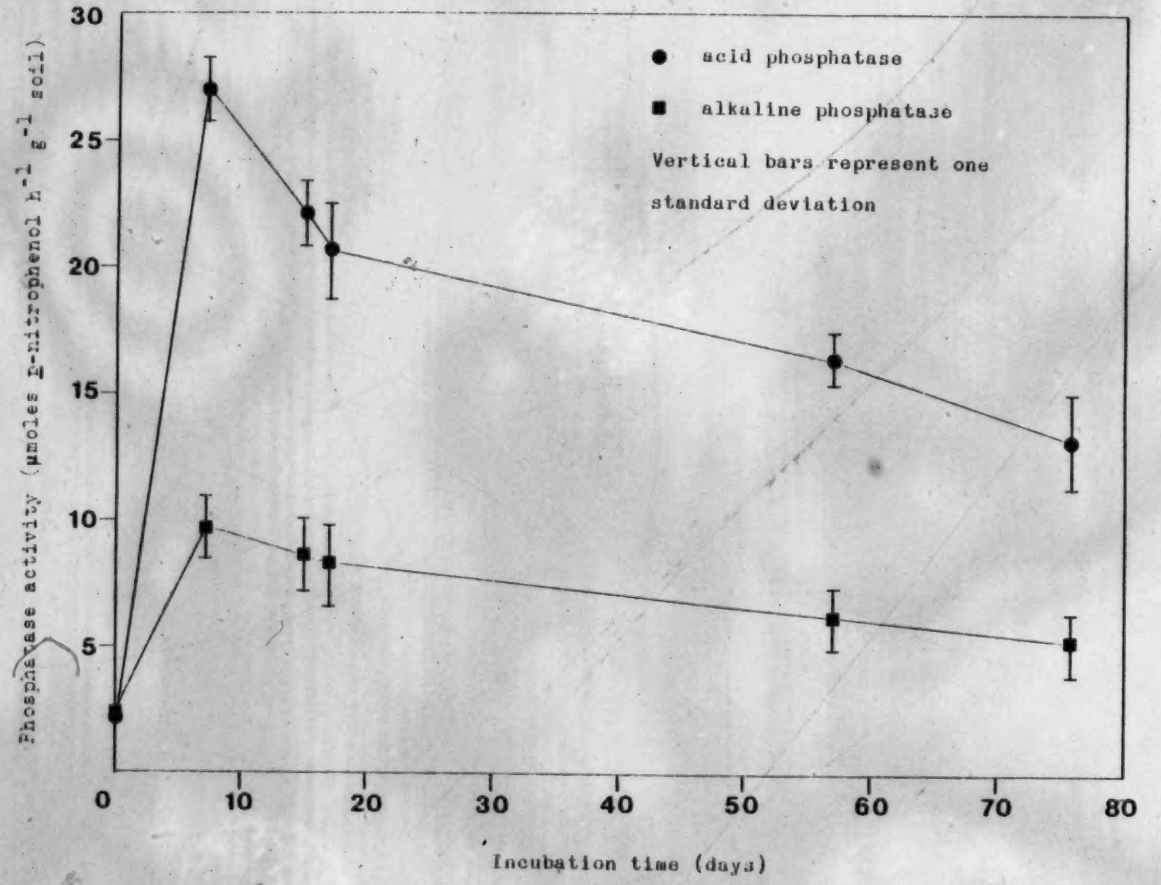


Figure 13. Phosphatase activity versus incubation time, treatment 2 (glucose and NH_4NO_3 in the first pulse), for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

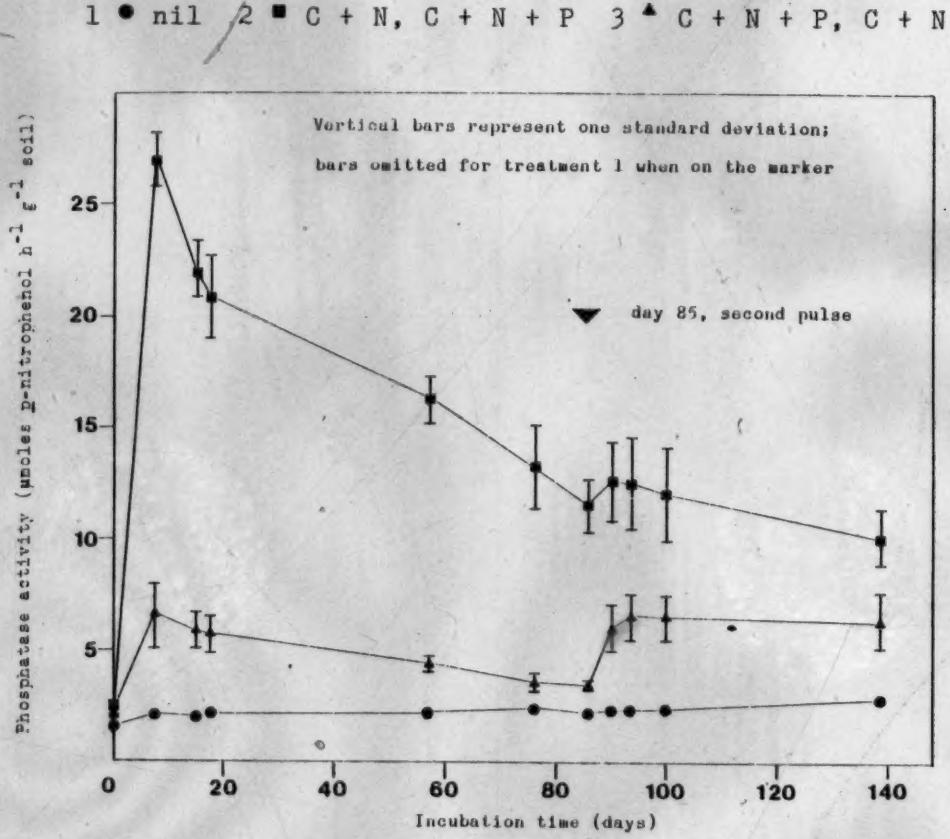


Figure 14. Acid phosphatase activity (pH 6.0) versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

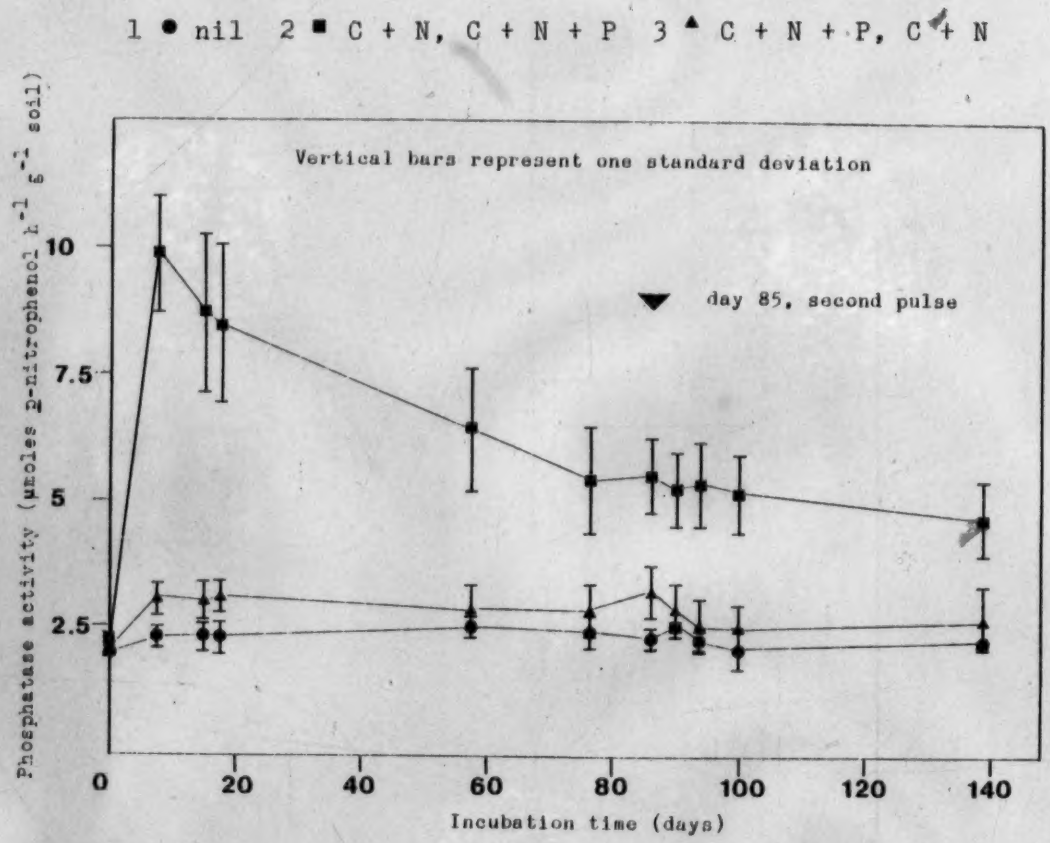


Figure 15. Alkaline phosphatase activity (pH 8.0) versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

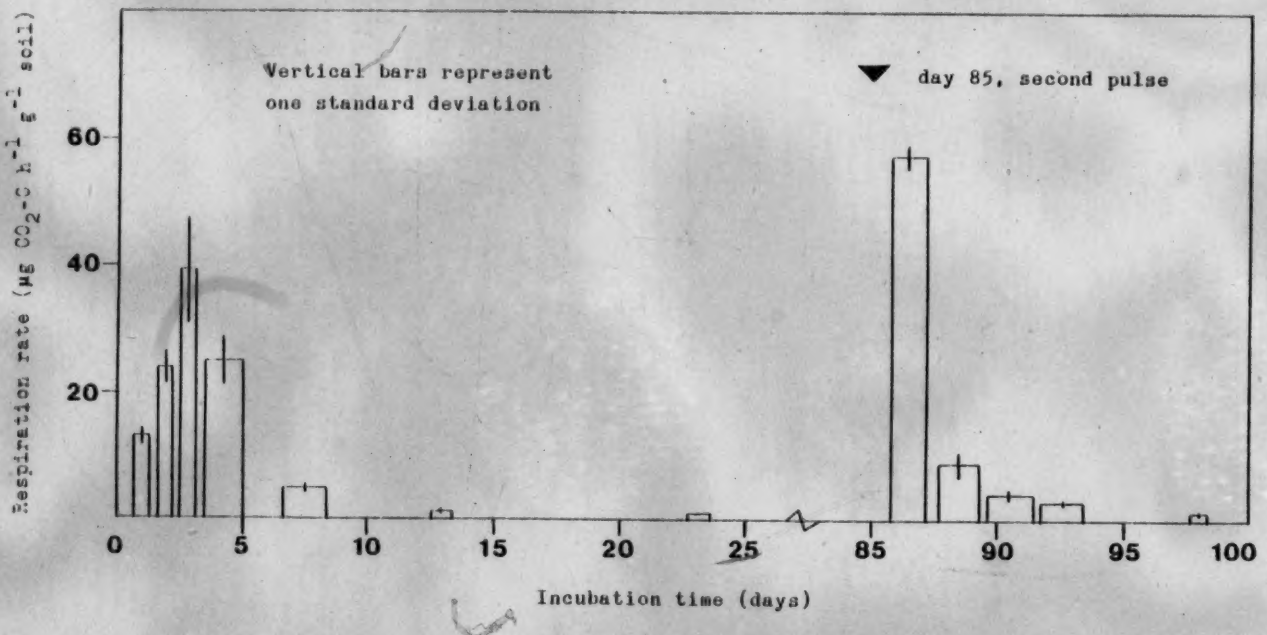


Figure 16. Respiration rate versus incubation time, treatment 2 (glucose and NH_4NO_3 in the first pulse, glucose, NH_4NO_3, and KH_2PO_4 in the second pulse), for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

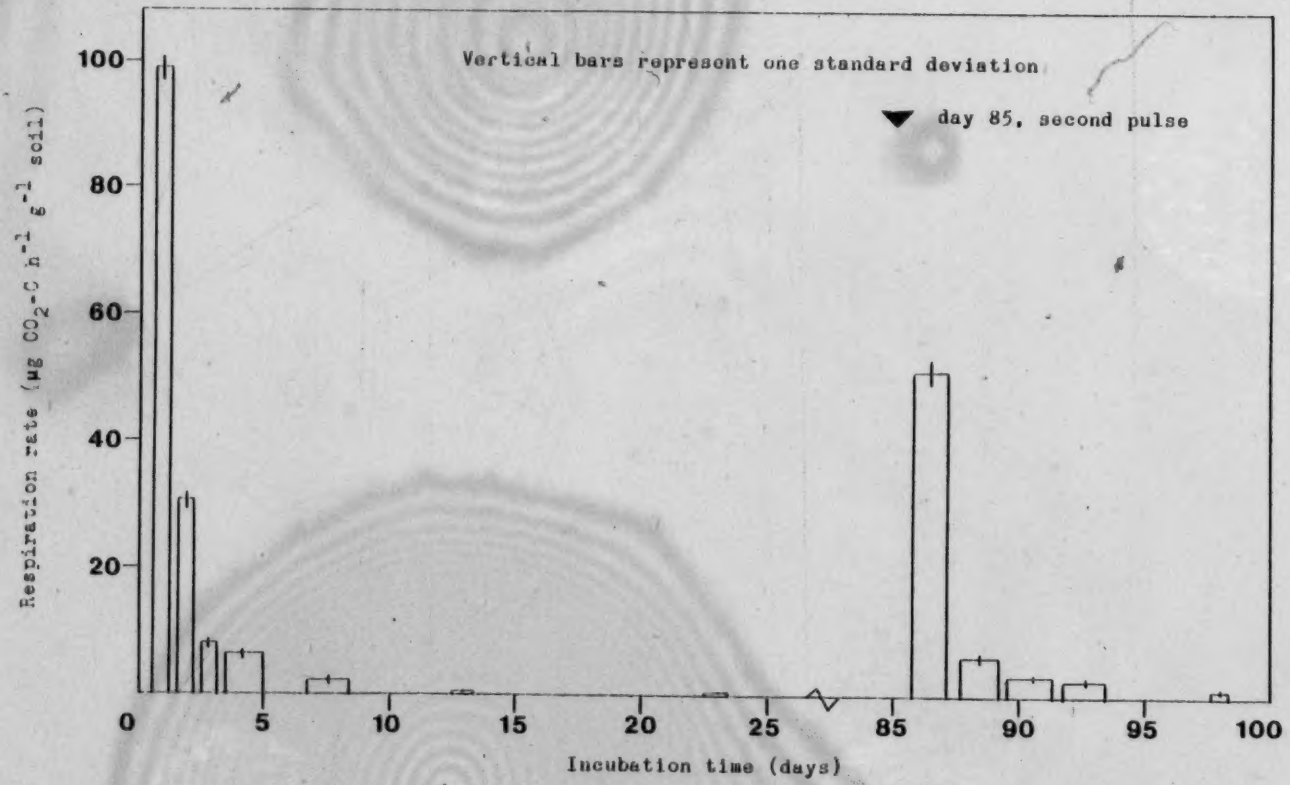


Figure 17. Respiration rate versus incubation time, treatment 3 (glucose, NH_4NO_3, and KH_2PO_4 in the first pulse, glucose and NH_4NO_3 in the second pulse), for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

phosphatase activity (especially in treatment 2) remained above the control level even after 70 days. Thus phosphatase was stabilized in the soil apart from a rapidly growing microbial population.

The measurements of available P extracted by 0.03 N NH_4F-H_2SO_4 and 0.5 M $NaHCO_3$ corroborate the phosphatase activity measurements. Both extracting solutions contained a high concentration of inorganic P for treatment 3 compared with the control (Figures 18 and 19). The level of inorganic P in both extracting solutions for treatment 2 was below the control level for most of the incubation period, reflecting derepressing conditions and immobilization of P. During incubation there was a rise in the level of inorganic P in the extracts of the two amended treatments, reflecting remineralization of P immobilized in microbial tissue during the early part of the experiment. This trend was much more pronounced in treatment 3. The $NaHCO_3$-extractable organic P measurements (Figure 20) show a high level of labile organic P in treatment 3 at day 1. The organic P fraction in $NaHCO_3$ extracts of soil does not represent P contained in microbial biomass, but is associated with microbial proliferation (Halm et al., 1972; Abbott, 1978; Bowman and Cole, 1978; Chauhan et al., 1979 and 1981). The subsequent decline in extractable organic P in treatment 3 reflects mineralization and adsorption of organic P compounds. Although, there was a large amount of microbial growth

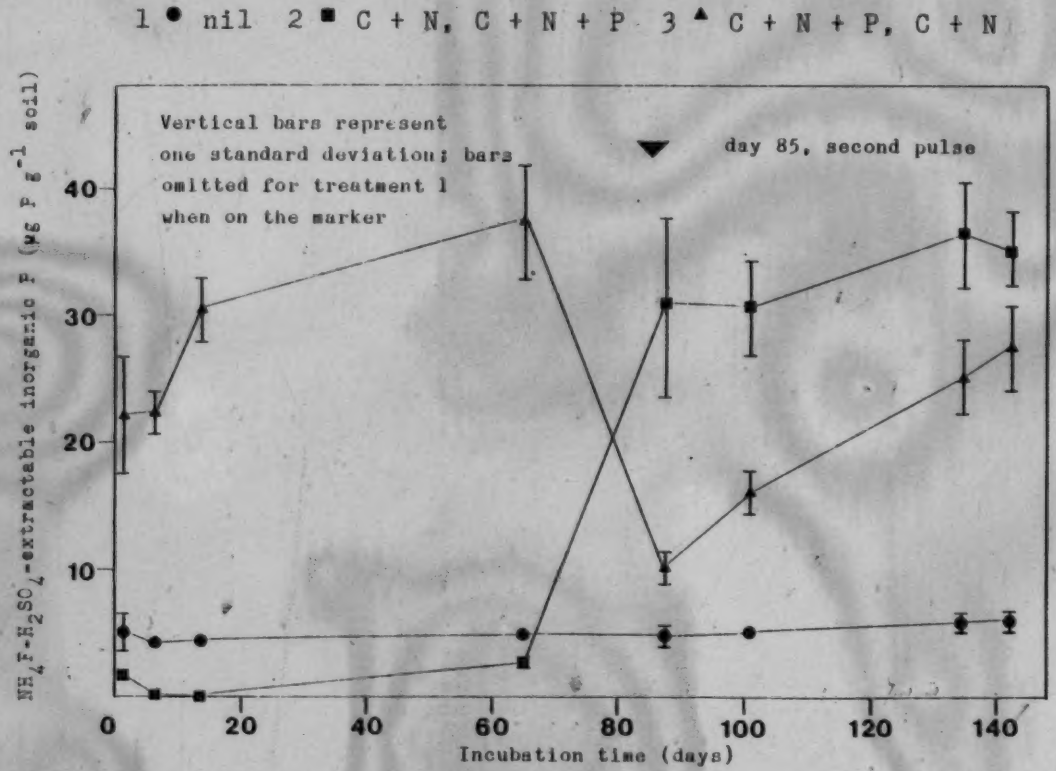


Figure 18. NH_4F-H_2SO_4-extractable inorganic P versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

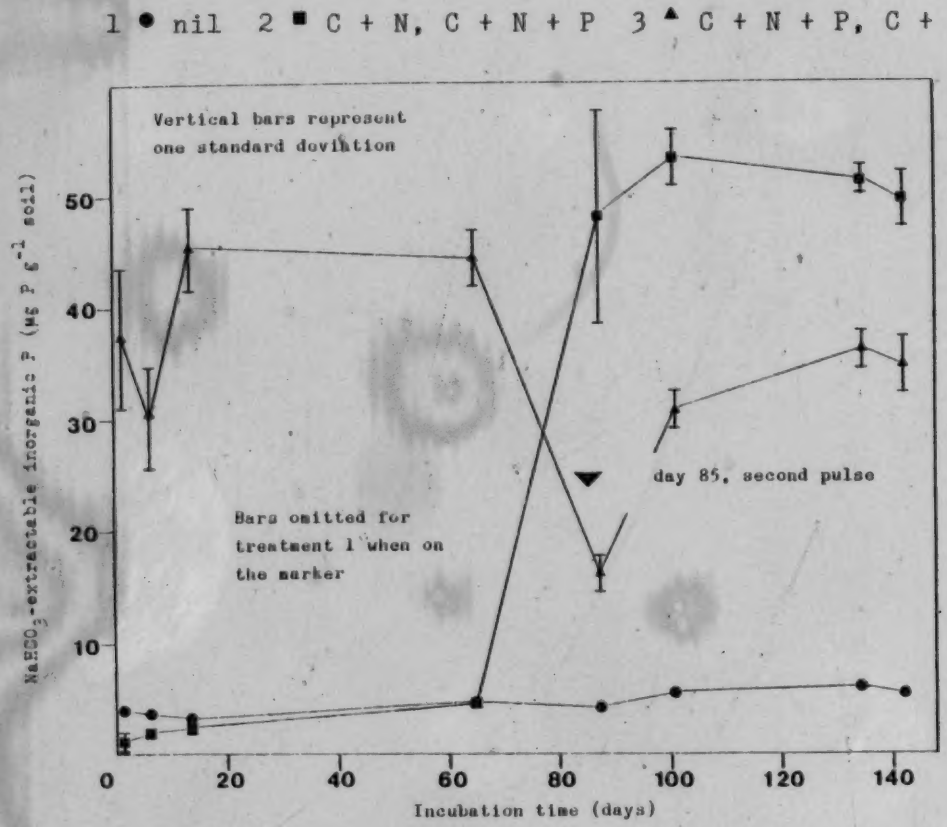


Figure 19. $NaHCO_3$-extractable inorganic P versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

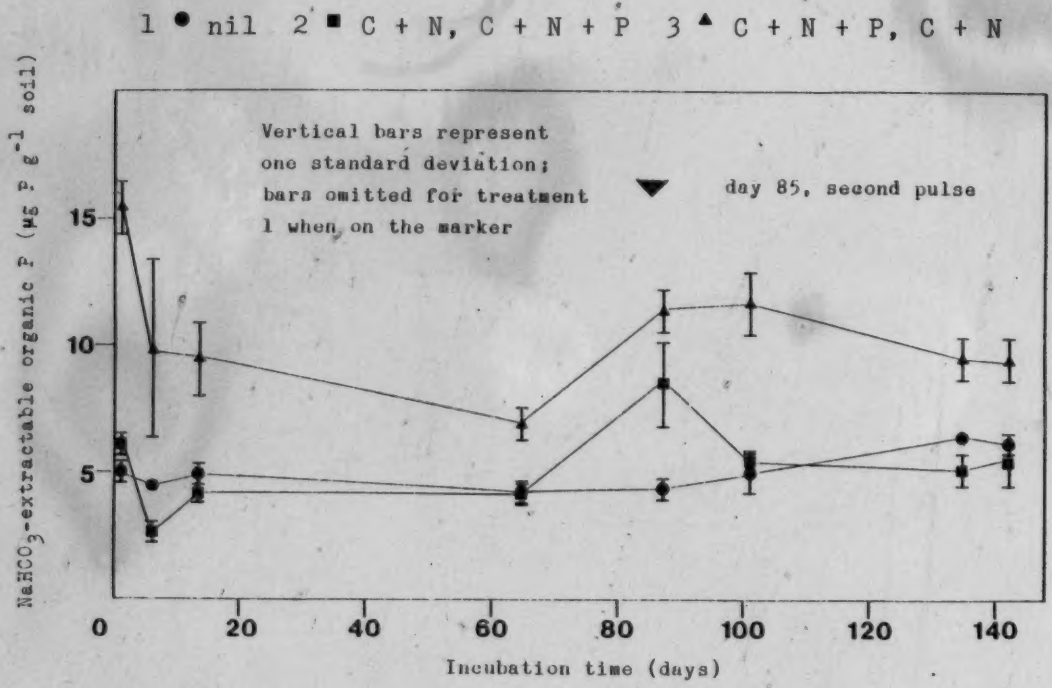


Figure 20. $NaHCO_3$-extractable organic P versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

and concomitant immobilization of P in treatment 2, there was no accumulation of $NaHCO_3$-extractable organic P (similar to that in treatment 3) at the beginning of the incubation period. Measurements of total $NaHCO_3$-extractable P (Figure 21) followed patterns similar to those of the inorganic P component. Because the total $NaHCO_3$-extractable P pool is dynamic over the course of incubation, changes in one component may not be quantitatively reflected in changes in the other component.

3.3.2 Second incubation period

The results of analyses following a second pulse of nutrients into the system must be considered in light of the data obtained during the first incubation period. The glucose and NH_4NO_3, and the glucose, NH_4NO_3, and KH_2PO_4 additions were switched in the second pulse, with the objective of observing the response in the amended soil samples to successive derepressing and repressing conditions in treatment 2, and successive repressing and derepressing conditions in treatment 3.

The phosphatase activity measurements (Figures 14 and 15) show that in the second incubation period for both amended treatments there was little or no change in the level of alkaline phosphatase activity. Addition of glucose and NH_4NO_3 in treatment 3 resulted in limited derepression of synthesis of acid phosphatase; there was only a small increase in phosphatase activity, un-

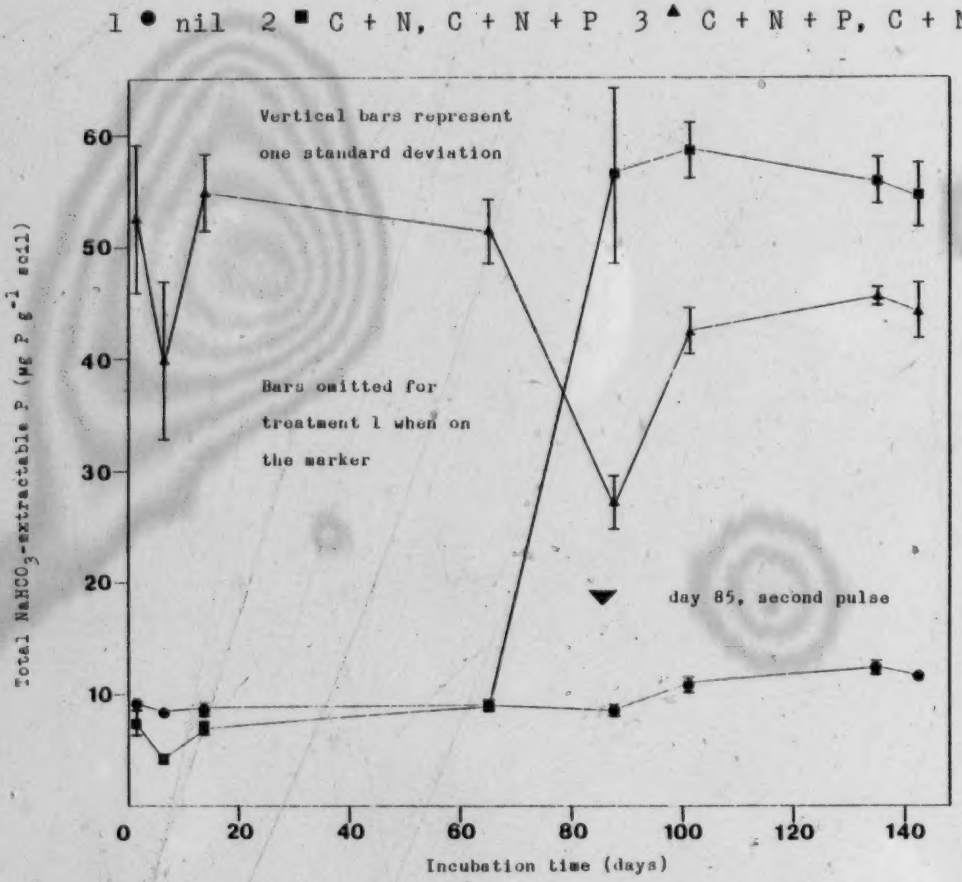


Figure 21. Total $NaHCO_3$-extractable P versus incubation time, for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in two pulses.

like the large peak of activity in the first incubation period. Addition of glucose, NH_4NO_3, and KH_2PO_4 in treatment 2 generally repressed synthesis of acid phosphatase; most of the activity in this treatment could be attributed to accumulated enzyme from the previous incubation period. These results confirm that acid phosphatase activity in the Breton soil is more responsive to derepressing conditions, and that production of new enzyme activity is more sensitive to P supply than is activity of existing enzyme.

The respiration data (Figures 16 and 17) following the second pulse of nutrients show a pattern in both amended treatments similar to that of treatment 3 in the first incubation period, with a rapid flush of microbial growth in response to addition of a readily oxidizable substrate. There was no delay in the peak respiration rate (as was observed in treatment 2 in the first incubation period), corroborating the results of the phosphatase activity measurements which show the absence of strong derepressing conditions in this incubation period. A slightly higher respiration rate was produced by treatment 2 relative to that in treatment 3.

The measurements of 0.03 N NH_4F-H_2SO_4- and 0.5 M $NaHCO_3$-extractable inorganic P show the changes resulting from the switch of the amendments in the second pulse (Figures 18 and 19). The addition of KH_2PO_4 in treatment 2 increased the level of inorganic P in both extracts

from that associated with the derepressing conditions of the first incubation period. In treatment 3 there was a large decrease in the level of inorganic P in both extracts, reflecting immobilization of residual KH_2PO_4 from the previous incubation period. It is significant, however, that the level of inorganic P in treatment 3 did not drop to the low level associated with the strong derepressing conditions produced by the addition of glucose and NH_4NO_3 in the first pulse of nutrients. This observation explains the absence of much derepression of phosphatase synthesis in the second incubation period. In both amended treatments, some remineralization of P initially immobilized during the flush of microbial growth following the second pulse of nutrients is indicated by the increase in extractable inorganic P during incubation. Change in extractable inorganic P is an insensitive indicator of mineralization because it reflects the net difference between mineralization, and precipitation and adsorption reactions, modified by the extraction solution. Further, the organic P fraction mineralized is not necessarily the extractable organic P component. There appeared to be greater mineralization during the second incubation period in treatment 3 than in treatment 2, although treatment 2 was associated with a higher level of phosphatase activity (mainly that of stabilized enzyme produced during the first incubation period). This observation, however, may be an artifact

of the extraction procedure.

In the second incubation period there was a small increase in $NaHCO_3$-extractable organic P in both amended treatments (Figure 20), associated with microbial proliferation. In treatment 2 there was a more rapid decline in the level of extractable organic P than in treatment 3, indicating a greater supply or slower turnover of the labile organic P fraction in treatment 3. Treatment 2, as stated above, produced a higher level of phosphatase activity. The measurements of total $NaHCO_3$-extractable P in the second incubation period again reflect the patterns of the inorganic P component (Figure 21). At the end of the experiment, there was a higher level of total extractable P in treatment 2 than in treatment 3, although both treatments received the same amount of KH_2PO_4. This observation indicates that at this time a larger amount of the added KH_2PO_4 remained immobilized in treatment 3 than in treatment 2, which produced the higher level of phosphatase activity.

3.4 Discussion

The results of this study generally agree with those reported in the literature describing phosphatase activity in incubated soil samples, and the controls on soil phosphatase activity. Nonetheless, they bring into question some of the earlier views which discount the

significance of soil phosphatase activity in mineralization of organic P compounds—particularly in soil receiving crop residues and in undisturbed ecosystems under a steady-state condition.

As described earlier, for each treatment nutrients were added to the soil in two pulses—one intended to derepress phosphatase synthesis, and the other intended to repress phosphatase synthesis. In treatment 2 phosphatase synthesis was initially derepressed by addition of glucose and NH_4NO_3, then repressed by added KH_2PO_4 (with glucose and NH_4NO_3) in the second incubation period. In treatment 3, however, residual KH_2PO_4 and recently synthesized phosphatase prevented substantial derepression of phosphatase synthesis by addition of glucose and NH_4NO_3 in the second pulse following repression during the first incubation period. These results support the conclusion of Spiers and McGill (1979) that the effect of orthophosphate on soil phosphatase activity is due more to its effect on enzyme synthesis than on activity of existing enzyme. Nannipieri *et al.* (1978) observed that addition of KH_2PO_4 (with glucose and $NaNO_3$) at four concentrations (0.05, 0.10, 0.15, and 0.30 mg P g^{-1} soil) repressed synthesis of acid phosphatase in incubated samples of two Dark Brown Chernozemic soils. The data obtained in the present study and that of Nannipieri *et al.* (1978) indicate that marked derepression of phosphatase synthesis by a proliferating microbial population

occurs only with low levels of labile inorganic P.

The initial rate of CO_2 evolution was increased in the first incubation period by added KH_2PO_4, indicating that the supply of soluble orthophosphate limited the microbial growth rate in the incubated soil for treatment 2. Similar results were obtained by Kramer and Yerdei (1959), Nannipieri et al. (1978), and Chauhan et al. (1981). Nannipieri et al. (1978) not only observed a higher initial rate of CO_2 evolution in incubated samples of a fine sandy loam amended with glucose, $NaNO_3$, and KH_2PO_4 (as opposed to samples which received glucose and $NaNO_3$), but also greater total CO_2 evolution in incubated samples of a clay similarly amended. Chauhan et al. (1981) also observed greater total CO_2 evolution in two incubated soils for the treatment in which KH_2PO_4 was included with cellulose and NH_4NO_3.

In the first incubation period the cumulative respiration measurements indicate that after 23 days approximately 44 and 40% of the added glucose-C remained in the incubated soil in treatment 2 and 3 respectively. In this study cumulative respiration measurements (as opposed to total respiration measurements obtained with an aeration train) provide only relative estimates of the amount of added glucose-C remaining. Nevertheless, the similarity in the amount of glucose-C remaining in the two amended treatments suggests that in treatment 2 mineralization of labile organic P or rapid desorption and dissolution of

labile inorganic P compensated for lack of added KH_2PO_4. Chauhan et al. (1981) reported that after 180 days of incubation of samples of a soil with low labile P status (3 µg $NaHCO_3$-extractable P g^{-1} soil), 57 and 53% of added cellulose-C remained in treatments receiving cellulose and NH_4NO_3, and cellulose, NH_4NO_3, and KH_2PO_4, respectively. The corresponding values for samples of a soil with high labile P status (18 µg $NaHCO_3$-extractable P g^{-1} soil) were 43 and 39%, without and with added KH_2PO_4, respectively. Chauhan et al. (1981) interpreted the similarity in values for amount of cellulose-C remaining as indirect evidence of mineralization of organic P in the treatment without added KH_2PO_4. Furthermore, in the present study the delayed peak in respiration measurements for treatment 2 fits the hypothesis that mineralization rather than desorption and dissolution was primarily responsible for increasing the orthophosphate supply in the absence of added KH_2PO_4.

As stated previously, in incubated samples of the Breton Ap horizon acid phosphatase production was more responsive to derepressing conditions than was production of alkaline phosphatase. On day 7 of the first incubation period, for treatment 2, acid phosphatase activity was 27.0 µmoles p-nitrophenol h^{-1} g^{-1} soil, while alkaline phosphatase activity was 9.8 µmoles p-nitrophenol h^{-1} g^{-1} soil. In a similar experiment Spiers and McGill (1979) observed a six-fold increase in acid phosphatase activity

(to levels greater than 80 umoles p-nitrophenol h^{-1} g^{-1} soil) in samples of a Black Chernozemic soil incubated with glucose and NH_4NO_3. Nannipieri et al. (1979) reported a large increase in acid phosphatase activity (to levels greater than 40 umoles p-nitrophenol h^{-1} g^{-1} soil) for samples of a red earth incubated with glucose and $NaNO_3$. In cases where there is a large increase in phosphatase activity in response to derepressing conditions, appreciable stabilization of enzyme may occur. In other soils, however, much smaller increases in acid phosphatase activity have been observed under derepressing conditions, and little stabilization of enzyme occurred (Nannipieri et al., 1978; Spiers and McGill, 1979). Hence soils vary in their capacity to produce phosphatase via a derepressed microbial population, and in their capacity to stabilize the enzyme produced. Much of this variation may be due to the original phosphatase level, the level of labile P, and past cultural practices. In the present study stabilization of newly synthesized phosphatase complicated the investigation of the controls on activity, particularly in the second incubation period. Furthermore, the present experiment did not distinguish between the significance of newly synthesized versus stabilized phosphatase in mineralization reactions.

For the Breton soil samples, $NaHCO_3$-extractable organic P measurements did not accurately assess labile

organic P. In further work labile P must be defined on a kinetic basis, and measurements of microbial P must be employed. In soil samples where microbial immobilization of P increases the size of the labile organic P fraction, inorganic P of $NaHCO_3$ extracts may partly reflect labile organic P. Abbott (1978) suggested that inorganic P of $NaHCO_3$ extracts may include labile organic P hydrolyzed during the 30-minute extraction period. This hypothesis may apply to $NaHCO_3$-extractable P measurements in the present study, particularly for treatment 2 which produced high levels of phosphatase activity in the first incubation period. Mineralization of labile organic P during the 30-minute extraction period would interfere with discrimination between labile inorganic and organic P, and may explain the absence of a large change in $NaHCO_3$-extractable organic P in the second incubation period. This hypothesis must be tested experimentally.

The results of the present study provide indirect evidence for the hypothesis that increased phosphatase production by microorganisms is associated with supply of P to a proliferating microbial population via mineralization of labile organic P. Several authors have, however, discounted the significance of phosphatase activity in situ. Bieleski (1973) stated that the significance of phosphatase activity in the external medium under natural conditions is uncertain because of the very low concentrations of phosphate esters in the medium. Tinker (1975)

suggested that because the organic P fraction of the soil solution is unlikely to be buffered to the same extent as the inorganic P fraction, the activity of root surface phosphatases is limited by the small amount of organic P reaching them. In the case of phosphatases which diffuse out into the soil from plant roots and soil microorganisms, orthophosphate produced by hydrolysis would be adsorbed and serve only to enlarge the labile inorganic P pool. Tinker (1975) further suggested that colloidal bacterial debris containing organic P compounds may be carried to the root surface in the transpiration stream, and be hydrolyzed there by surface phosphatases. In such a situation, reasonably abundant supplies of organic P may be available. These hypotheses indicate that in assessing the significance of phosphatase activity *in situ*, its role in supplying P to plants must be considered separately from its role in supplying P to microorganisms (and thence the labile P pool).

Further evidence for the ecological significance of soil phosphatase activity was presented by Cole *et al.* (1977) who developed a simulation model of the P cycle in semiarid grasslands. Flow rates for uptake of P by decomposers were derived from estimated seasonal populations of decomposers, turnover rates, and scattered data on P composition of various decomposer species. The model predicted that plant and decomposer uptake from the labile inorganic P pool is balanced by replenishment of this

pool mainly by mineralization of labile organic P. For the data set of the Pawnee (Colorado) site, simulation results indicated a complete turnover of labile inorganic P during one season. For the Matador (Saskatchewan) site, the model indicated a four-fold turnover of this pool during the season. It is significant that P uptake by microbial and other decomposers was the largest annual flow of P in the model; at the Pawnee site simulation results indicated that decomposer uptake was nearly five times greater than plant uptake. Cole et al. (1978), in a study of immobilization and mineralization of P in incubated soil simulating rhizospheres, observed that bacteria quickly immobilized much of the labile inorganic P during metabolism of carbon substrates. Most of the bacterial P was subsequently mineralized and released into the soil during predation by amoebae. If during derepressed growth on readily oxidizable substrates the proliferating microbial population supplies its P requirements by synthesis of phosphatase and mineralization of organic P compounds, subsequent release of P immobilized in microbial biomass may result in an indirect transfer of P from the organic to the labile inorganic pool.

3.5 Conclusions

The results of the present study support the following conclusions:

(i) synthesis of acid phosphatase by the proliferating microbial population in incubated samples of the Breton Ap horizon was more responsive to derepressing conditions than was synthesis of alkaline phosphatase.

(ii) studies of the controls on phosphatase production in incubated soil samples are more complicated than chemostat studies of microorganisms grown in solution culture because a significant amount of the phosphatase produced in amended soil by microorganisms is stabilized and remains active.

(iii) although the extractable P measurements detected changes in the level of labile inorganic P associated with repression and derepression of microbial synthesis of phosphatase, they did not clearly establish the role of recently synthesized enzyme in mineralization.

(iv) for treatment 3 (soil receiving glucose, NH_4NO_3, and KH_2PO_4 in the first pulse, and glucose and NH_4NO_3 in the second), residual added KH_2PO_4 and recently synthesized phosphatase prevented marked derepression of phosphatase synthesis (by addition of glucose and NH_4NO_3 in the second pulse) following repression in the first incubation. This

observation suggest that in nature appreciable de-repression of phosphatase synthesis by micro-organisms occurs with the addition of a readily oxidizable substrate in soil with a low level of labile inorganic P and little recently synthesized enzyme.

Further research is required with specific attention to several problems:

(i) the factors which determine the size of the labile organic P pool (the concentration of organic P compounds in the soil solution and the recharge capacity of the soil with respect to that pool) need to be identified.

(ii) the portion of soil organic P accessible for hydrolysis by phosphatase needs to be identified, and the effect of spatial and temporal variation of substrate supply on soil phosphatase activity <u>in situ</u> must be examined.

(iii) the hypothesis that soil phosphatase activity can affect the size of the labile inorganic P pool by increasing the recharge capacity needs to be examined.

(iv) the effect of additions of a microbial energy source such as crop residues on immobilization of added and native P in microbial biomass, and sub-

sequent release of immobilized P to the labile inorganic pool requires further investigation.

Determination of Organic P in $NaHCO_3$ Extracts of Soil by a Persulfate Digestion Method

Klingaman and Nelson (1976) reported that the best technique for preservation of unfiltered samples of river water, surface runoff, and tile drainage water (containing 0.030-0.96 mg P l^{-1}) prior to analysis for soluble inorganic P was storage at subzero temperatures. They noted, however, that freezing of samples containing sediments may decrease the recovery of soluble inorganic P. Hence, in the present study the 0.5 M $NaHCO_3$ and 0.03 N NH_4F-H_2SO_4 extracts of the incubated soil samples were frozen immediately after filtration to await analysis for inorganic and organic P.

Various workers (Bowman and Cole, 1978; Cole et al., 1978; Chauhan et al., 1979) have used a nitric-perchloric acid digestion technique to determine organic P in $NaHCO_3$ extracts of soil. Because proper facilities for the safe use of perchloric acid were not available in the present study, an alternative digestion procedure was required. Gales et al. (1966) developed a persulfate digestion method for determination of total P in surface waters. The effectiveness of the method was tested with selected inorganic and organic P compounds (sodium metaphosphate, sodium pyrophosphate, sodium tripolyphosphate, glucose-1-

phosphate, fructose-6-phosphate, sodium ß-glycerophosphate, sodium deoxyribonucleate, lecithin, calcium phytate, and adenosine-5'-monophosphate), algal suspensions, and filtered and unfiltered water samples. There was essentially quantitative recovery of phosphate from all three inorganic salts, and all of the organic P compounds except adenosine-5'-monophosphate, for which the recovery of phosphate was 84-100%. The method gave consistent recovery of phosphate from algae suspensions in which there was an artificially high level of organic matter to be oxidized, and showed that a substantial amount of P was contained in the suspended portion of unfiltered water samples. Carter et al. (1974) used the persulfate digestion method for determination of total P in filtered and unfiltered samples of irrigation and surface drainage water. When compared with the nitric-perchloric acid digestion and Na_2CO_3 fusion methods for total P, their method consistently gave values as high as, or slightly higher than those obtained by the other two procedures, and was more precise. The persulfate digestion method was also used to determine total P in the sediments of the unfiltered water samples.

A persulfate digestion method (Rand et al., 1976, p. 476) was adapted for determination of organic P in $NaHCO_3$ extracts of soil as follows:

(i) 25-ml aliquots of $NaHCO_3$ extracts were pipetted into 125-ml Erlenmeyer flasks; 3 ml of 5 N H_2SO_4 were

added to neutralize the extract, followed by 25 ml of deionized water.

(ii) 1 ml of digest solution and 0.4 g of potassium persulfate ($K_2S_2O_8$) were added to each flask, and the samples were boiled gently on a hot plate for at least 40 minutes.

(iii) the contents of each flask were cooled, transferred with rinsings to a 100-ml volumetric flask, neutralized to a light pink color with 1 N NaOH and phenolphthalein indicator solution, and then made up to 100 ml with deionized water.

(iv) inorganic P in the digest solutions was determined by the method of Watanabe and Olsen (1965) with absorbance measurements at 730 nm. After correction for blanks (containing 25 ml of 0.5 M $NaHCO_3$ solution instead of extract), values of total P for the extracts were obtained from a standard curve for solutions of inorganic P (giving final concentrations of 0.25, 0.50, 1.0, 1.5, 2.0, and 2.5 ppm) which had been carried through the digestion procedure. Organic P was taken as the difference between total and inorganic P.

Abbott (1978) determined organic P in five extracts of calcareous soil as the difference in total P (by digestion with H_2O_2, NaClO, or NaBrO) and inorganic P. Extractable total P values were poorly correlated with each other, indicating differences in the nature

of the organic P extracted by different procedures, and in the nature of the organic P converted to inorganic form. Hence, in the present study the organic P measurements may differ from values obtained with the nitric-perchloric acid digestion method which is generally considered to be more rigorous (Rand et al.,1976, p. 474). Nevertheless, the persulfate digestion method should account for the labile organic P compounds in the $NaHCO_3$ extract.

Experimental Data

Treatment 1 - moistened soil (control)

Treatment 2 - soil receiving C (5 mg glucose-C g^{-1} soil) plus N (438 µg NH_4NO_3-N g^{-1} soil) in the first pulse, and C plus N (as above) plus P (200 µg KH_2PO_4-P g^{-1} soil) in the second pulse

Treatment 3 - soil receiving C plus N plus P (as above) in the first pulse, and C plus N (as above) in the second pulse

Table 1. Acid phosphatase activity measurements for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, μmoles p-nitrophenol h^{-1} g^{-1} soil).

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
0	1.6 ± 0.10	2.3 ± 0.24	1.9 ± 0.27
7	2.1 ± 0.10	27.0 ± 1.2	6.6 ± 1.4
15	2.2 ± 0.23	22.1 ± 1.3	5.9 ± 0.80
17	2.2 ± 0.21	20.7 ± 1.9	5.8 ± 0.70
57	2.4 ± 0.19	16.3 ± 1.1	4.4 ± 0.39
76	2.5 ± 0.13	13.2 ± 1.9	3.8 ± 0.42
Period 2			
0	2.3 ± 0.25	11.6 ± 1.3	3.7 ± 0.21
5	2.5 ± 0.28	12.7 ± 1.8	6.0 ± 1.0
9	2.5 ± 0.30	12.6 ± 2.0	6.5 ± 1.1
15	2.5 ± 0.17	12.0 ± 2.1	6.5 ± 1.0
54	2.8 ± 0.17	10.0 ± 1.1	6.4 ± 1.3

Table 2. Alkaline phosphatase activity measurements for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, μmoles p-nitrophenol h^{-1} g^{-1} soil).

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
0	2.0 ± 0.17	2.3 ± 0.20	2.1 ± 0.13
7	2.3 ± 0.22	9.8 ± 1.2	3.1 ± 0.31
15	2.3 ± 0.28	8.7 ± 1.5	2.9 ± 0.40
17	2.3 ± 0.35	8.4 ± 1.6	2.9 ± 0.40
57	2.5 ± 0.25	6.4 ± 1.2	2.9 ± 0.49
76	2.4 ± 0.19	5.4 ± 1.1	2.8 ± 0.55
Period 2			
0	2.2 ± 0.21	5.5 ± 0.73	3.1 ± 0.46
5	2.5 ± 0.05	5.2 ± 0.73	2.8 ± 0.52
9	2.3 ± 0.22	5.3 ± 0.78	2.6 ± 0.49
15	2.1 ± 0.46	5.1 ± 0.84	2.5 ± 0.40
54	2.2 ± 0.05	4.6 ± 0.74	2.7 ± 0.62

Table 3. Respiration measurements for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, μg CO_2-C h^{-1} g^{-1} soil).

Day*	Treatment 1	Treatment 2	Treatment 3
Period 1			
1	0	13.2 ± 1.1	98.7 ± 1.9
2	0	23.9 ± 2.6	30.8 ± 1.4
3	0	39.3 ± 8.5	8.5 ± 1.1
5	0	24.9 ± 4.1	6.7 ± 0.47
8	0.19 ± 0.14	5.1 ± 0.46	2.6 ± 0.30
13	0	1.2 ± 0.27	0.82 ± 0.27
23	0.11 ± 0.12	0.70 ± 0.09	0.53 ± 0.13
Period 2			
2	0	57.3 ± 1.9	51.2 ± 2.3
4	0	8.7 ± 2.0	6.3 ± 0.71
6	0	4.0 ± 0.45	3.1 ± 0.54
8	0	2.5 ± 0.37	2.2 ± 0.27
13	0	1.3 ± 0.24	1.2 ± 0.27

* day on which measurement period ended

Table 4. Inorganic P extracted in 0.03 N NH_4F-H_2SO_4 for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, μg P g^{-1} soil).

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
1	4.9 ± 1.5	1.5 ± 0.45	22.1 ± 4.7
7	4.1 ± 0.48	0	22.2 ± 1.9
14	4.4 ± 0.25	0	30.5 ± 2.6
65	4.9 ± 0.62	2.8 ± 0.23	37.4 ± 4.3
Period 2			
2	4.7 ± 0.66	30.4 ± 7.0	10.3 ± 1.4
16	5.2 ± 0.70	30.5 ± 3.7	15.8 ± 1.7
50	6.0 ± 0.56	36.2 ± 4.0	25.2 ± 2.7
57	6.1 ± 0.57	35.2 ± 3.2	27.8 ± 3.3

Table 5. Inorganic P extracted in 0.5 M $NaHCO_3$ for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, ug P g^{-1} soil).

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
1	4.2 ± 0.46	1.2 ± 0.51	37.3 ± 6.2
7	3.8 ± 0.24	1.8 ± 0.34	30.0 ± 4.6
14	3.6 ± 0.25	2.5 ± 0	45.2 ± 3.7
65	5.0 ± 0.43	4.9 ± 0.41	44.3 ± 2.7
Period 2			
2	4.3 ± 0.29	47.9 ± 9.5	15.9 ± 1.6
16	5.5 ± 0.31	53.3 ± 2.2	30.7 ± 1.6
50	6.1 ± 0.12	51.4 ± 1.6	36.2 ± 1.5
57	5.8 ± 0.25	49.4 ± 2.4	34.6 ± 2.5

Table 6. Organic P extracted in 0.5 M $NaHCO_3$ for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as decribed above ($\bar{X} \pm s$, $\mu g\ P\ g^{-1}$ soil.)

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
1	5.1 ± 0.46	6.1 ± 0.58	15.3 ± 0.86
7	4.4 ± 0.26	2.6 ± 0.50	9.9 ± 3.3
14	4.9 ± 0.41	4.2 ± 0.29	9.5 ± 1.4
65	4.2 ± 0.34	4.1 ± 0.35	7.0 ± 0.63
Period 2			
2	4.4 ± 0.41	8.6 ± 1.6	11.3 ± 0.86
16	4.9 ± 0.65	5.4 ± 0.39	11.7 ± 1.2
50	6.3 ± 0.28	4.9 ± 0.74	9.5 ± 0.96
57	6.2 ± 0.25	5.3 ± 0.83	9.4 ± 0.90

Table 7. Total P extracted in 0.5 M $NaHCO_3$ for samples of the Breton Ap horizon incubated at field capacity and 22-25 C following amendment in 2 pulses as described above ($\bar{X} \pm s$, μg P g^{-1} soil.)

Day	Treatment 1	Treatment 2	Treatment 3
Period 1			
1	9.3 ± 0.57	7.3 ± 1.0	52.6 ± 6.6
7	8.2 ± 0.30	4.4 ± 0.68	39.9 ± 7.1
14	8.5 ± 0.62	6.7 ± 0.29	54.7 ± 3.4
65	9.2 ± 0.57	9.0 ± 0.66	51.3 ± 3.0
Period 2			
2	8.7 ± 0.68	56.5 ± 8.0	27.2 ± 2.1
16	10.4 ± 0.93	58.6 ± 2.4	42.3 ± 1.9
50	12.4 ± 0.38	56.2 ± 2.2	45.7 ± 1.1
57	11.9 ± 0.33	54.6 ± 2.9	44.1 ± 2.5

CHAPTER 4

EFFECT OF CROP, ROTATION, AND FERTILIZER ON SOIL PHOSPHATASE ACTIVITY IN THE FIELD

4.1 Introduction

4.1.1 The nature and role of plant root phosphatase production

In the plant-soil system the role and significance of phosphatase production is not well-defined. Repression of enzyme synthesis and inhibition of activity at the microsite do not by themselves adequately describe control of phosphatase levels in the field. Plant roots produce phosphatase (Rogers et al., 1942; Estermann and McLaren, 1961; Woolhouse, 1969; Ridge and Rovira, 1971; Gould et al., 1979), and plants can utilize organic P esters as the sole source of P in solution culture, or as a primary source of P in soil (Rogers et al., 1942; Wild and Oke, 1966; Sen Gupta and Cornfield, 1967; McKercher and Tollefson, 1978). These two facts together with the demonstrated repression of plant root phosphatase synthesis and inhibition of root phosphatase activity by orthophosphate (Woolhouse, 1969; Bieleski and Johnson, 1972) support the hypothesis that the purpose of phosphatase production by plant roots is to utilize soil organic P.

Nevertheless, hydrolysis of soil organic P esters by plant root phosphatases has not been demonstrated. Alternate hypotheses suggest that plant root phosphatase production facilitates utilization of P esters released to the medium by dying plants (Bieleski and Johnson, 1972), or results from a greater proportion of senescing tissue and a higher turnover rate of absorbing roots on P-deficient sites (Alexander and Hardy, 1981).

Woolhouse (1969) presented evidence for physiological adaptation of root phosphatase activity to the chemical environment in soil, in addition to response of root phosphatase synthesis to the solution concentration of orthophosphate. He determined the effect of various concentrations of Al, Ca, and Pb on surface acid (pH 4.5) p-nitrophenyl phosphatase activity of excised root tips of three races of Agrostis tenuis—acid soil race, calcareous soil race, and lead spoil race. Phosphatase activity of root tips from plants of calcareous soil and lead spoil races was inhibited to a greater degree by low concentrations of Al than activity of root tips of plants of the acid soil race. Similarly, Ca and Pb had their greatest inhibitory effects on phosphatases of races which grew in soils which did not contain high concentrations of these ions. These results together with those for ATP-ase activity of cell wall preparations of roots suggest an apparent difference among enzymes of each race in terms of interactions with particular metal

ions. It was concluded that differences among edaphic ecotypes of A. tenuis with respect to phosphatase activity of the root surface may result from active evolution of the structure of the phosphatase enzymes in response to the ionic composition of the soil solution.

4.1.2 Microbial phosphatase production in the rhizosphere

In the rhizosphere both microorganisms and plant roots produce phosphatase. Estermann and McLaren (1961) studied the contribution of rhizosphere microorganisms to phosphatase activity of roots of barley plants, and concluded that although nonsterile roots possessed higher activity than sterile roots, much of the phosphatase activity of the root zone is that of enzyme produced by the root. Similarly Saxena (1964) reported that nonsterile roots of pea, gram, wheat, and barley possessed higher phytase activity than sterile roots. Greaves and Webley (1965) reported greater total numbers of microorganisms capable of decomposing selected organic phosphates (phenolphthalein diphosphate, sodium glycerophosphate, sodium phytate, lecithin, and ribonucleic and deoxyribonucleic acid) in the rhizosphere of three pasture grasses relative to nonrhizosphere soil. Moreover, occasionally preferential stimulation of microorganisms capable of decomposing some of the substrates was observed in the rhizosphere. Gould et al. (1979) reported that

the presence of bacteria or bacteria and amoebae together increased the amount of acid phosphatase in solution around roots of Bouteloua gracilis. The K_m and heat stability of the additional activity were identical to those of the plant acid phosphatase. Furthermore, plant phosphatase synthesis was not derepressed by low orthophosphate levels resulting from microbial uptake. They therefore concluded that plant roots were the primary source of additional phosphatase activity in the culture solutions containing the microorganisms. These results support the hypothesis of increasing levels of phosphatase in the rhizosphere because of plant production of enzyme together with increased microbial production of enzyme.

In other instances the presence of microorganisms in the rhizosphere has not increased phosphatase activity. Ridge and Rovira (1971) reported that inoculation of roots of intact wheat seedlings with pure cultures of bacteria and a fungus known to possess phosphatase activity did not increase root phosphatase activity. Moreover, in some cases there was reduced activity in the presence of microorganisms, which could not be attributed to sorption of substrate or product by microorganisms.

Investigations of the significance of microbial phosphatase production in the rhizosphere suggest that it increases microbial rather than plant uptake of P. For example, Szember (1960) reported no significant improvement in growth of radish plants from inoculation of

lecithin- or phytin-decomposing microorganisms onto roots of plants supplied with lecithin or phytin, respectively, as the sole source of P, over that obtained with plants grown under sterile conditions. Martin and Cartwright (1971) used the ^{32}P count of leaves to show that ^{32}P myo-inositol hexaphosphate ($IH^{32}P$) was equivalent to $KH_2{}^{32}PO_4$ in supplying P to perennial ryegrass (Lolium perenne) in a soil with low P retention at 200 ppm P but not at 20 ppm P. Their method assessed plant uptake and translocation of ^{32}P following mineralization of substrate. Using the same method, Martin (1973) demonstrated that in solution culture the presence of large numbers of microorganisms (with or without phytase activity) in the root region of wheat plants (Triticum aestivum) had no effect on the amount of ^{32}P activity translocated to plant tops from $IH^{32}P$. Similarly, with plants grown in soil containing $IH^{32}P$ at 0.2 ppm P, inoculation of the root region with bacteria possessing phytase activity or a mixed rhizosphere microflora had no effect on the amount of ^{32}P activity recovered in plant tops. Although Martin (1973) concluded that the low concentration of inositol hexaphosphate in the soil solution prevented increased hydrolysis of phytate by microbial phosphatase, his results also support the hypothesis that plant uptake of orthophosphate is not directly increased by hydrolytic activity of microbial phosphatase in the root region.

4.1.3 Complexity of plant-microbe interactions in the rhizosphere

Rhizosphere microorganisms affect orthophosphate availability to the root by means other than phosphatase production. Barber (1966) reported that microorganisms on roots of barley (Hordeum vulgare) had a marked effect on absorption of labelled inorganic P from dilute solutions. At a low concentration of P (0.001 ppm), microorganisms influenced both uptake and translocation of P, so that roots of sterile plants contained almost twice as much P as those of nonsterile plants, and the amount transferred to the shoots was nearly 20 times greater. Because most of the ^{32}P assimilated by nonsterile plants was present as nucleic acid P, Barber (1966) concluded that microorganisms associated with roots of nonsterile plants had preferentially absorbed P from dilute solutions. Conversely, Bowen and Rovira (1966) reported that at a low concentration of labelled inorganic P (0.005 mM), P uptake in tomato (Lycopersicon esculentum) and clover (Trifolium subterraneum) plants was 77-95 and 55-60% higher, respectively, in nonsterile versus sterile seedlings. Furthermore, translocation of P to plant tops in tomato seedlings over 20 minutes was 4.4 times higher in nonsterile plants, and P translocation in clover seedlings showed a similar pattern. They concluded that increased P uptake by nonsterile plants was not solely due to absorption by rhizosphere microorganisms, but included

direct effects of microorganisms on plant metabolism.

Various soil microorganisms solubilize insoluble inorganic P compounds (Das, 1963; Subba-Rao and Bajpai, 1965; Chhonkar and Subba-Rao, 1967; Hmeidan, 1982). Katznelson *et al*. (1962) reported that absolute numbers of P-dissolving bacteria were much greater in rhizosphere soil than in root-free soil for most of the plant species examined. They did not, however, obtain strong evidence for a selective effect of plant roots on P-dissolving microorganisms. Das (1963) hypothesized that following incorporation of P from insoluble salts into biomass by fungi, subsequent death and decomposition of microorganisms may release P to the labile pool.

The literature indicates that in soil the influence of phosphatase activity of plant roots and microorganisms on orthophosphate availability to the root is difficult to assess because of the complexity of interactions between plant roots and microorganisms.

4.1.4 Soil phosphatase activity measurements on samples from the Breton Plots

The present study examined phosphatase activity of soil samples from the Breton Plots on a Gray Luvisolic soil in Alberta. Khan (1970) investigated the effect of four fertilizer treatments (manure, NPKS, NS, and nil) on soil phosphatase activity (towards phenyl phosphate at pH 7.0) of samples collected in 1969 from the Ap

horizon of two rotations of the Breton Plots. His results represent the effects of cropping systems and fertilizers on soil phosphatase activity 39 years after establishment of the plots, and 10 years prior to the present study. Phosphatase activity in soil samples of the five-year rotation of grains and legumes was significantly higher than that in soil samples from the wheat-fallow system. Higher phosphatase activity was observed for plots which received manure, NPKS, or NS compared with the control, but the differences were not statistically significant. The magnitude of the increases in phosphatase activity due to fertilizers was greater with the five-year rotation than with the wheat-fallow system. The five-year rotation also produced a higher organic matter content than the wheat-fallow system. The higher levels of organic matter and phosphatase activity of the five-year rotation may result independently from increased plant productivity, or higher organic matter content may increase stabilization of phosphatase produced.

4.1.5 Conclusions and experimental objectives

The literature reviewed indicates that:

(i) plants can use organic phosphates as the sole source of P in solution culture under sterile conditions via action of root phosphatases.

(ii) although orthophosphate has been shown to

inhibit activity of root phosphatase, and repress synthesis of enzyme by the root, such controls have not been clearly linked to plant utilization of soil organic P.

(iii) phosphatase activity in the rhizosphere may be increased by the presence of microorganisms over that due to plant enzyme. Increased activity, however, does not necessarily increase plant uptake of P. Rather, it may increase microbial uptake of P.

(iv) the effect of plant and microbial production of phosphatase in the rhizosphere is difficult to assess because of other plant-microorganism interactions such as competition between roots and microorganisms for orthophosphate, microbial effects on plant physiology, and solubilization of insoluble inorganic P by microorganisms.

(v) the effect of fertilizer P on soil phosphatase activity (in bulk samples from the field) cannot be explained solely in terms of controls of enzyme activity and synthesis by orthophosphate at the microsite.

(vi) patterns of seasonal variation of phosphatase activity and available P in the field may or may not be similar. Increased soil phosphatase activity may increase or decrease specific fractions of soil organic P.

The objective of this particular study was to examine temporal and spatial variation of phosphatase activity (representing enzyme level) in bulk samples of soil from the field without discrimination between rhizosphere and nonrhizosphere soil. Furthermore, the effect of crop, rotation, and fertilizer (especially inorganic P) on enzyme levels in bulk soil samples was related to controls on phosphatase activity by orthophosphate which operate at the microsite. The two hypotheses tested were that plant growth over the growing season increases soil phosphatase activity, and that application of inorganic P fertilizer decreases soil phosphatase activity by repression of enzyme synthesis by plant roots and microorganisms in the field. The results of this study will suggest hypotheses about controls on mineralization of labile organic P in the field, and its significance (directly or indirectly) in supply of P to soil microorganisms and plants.

4.2 Materials and Methods

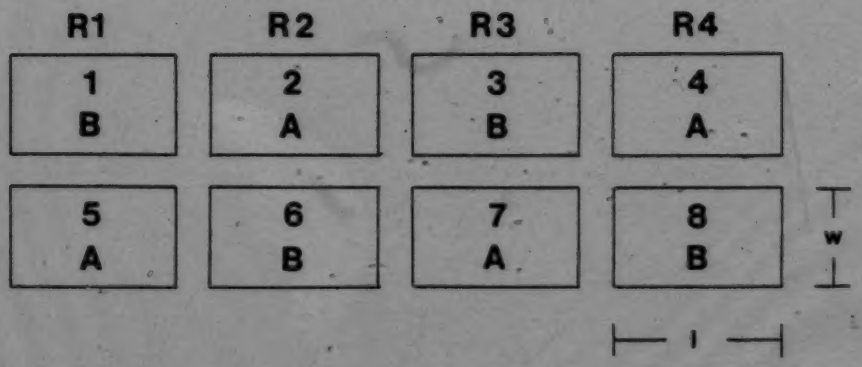
4.2.1 Soils

Soil samples were collected at two field sites—one near Ellerslie at the experimental farm of the Department of Soil Science of the University of Alberta (NE-24-51-25 W4) on a Malmo SiCL soil; the other at the Breton Plots (NE-25-47-4 W5) on a Breton L-SiL soil.

4.2.2 Ellerslie site

The field study on the Malmo soil was conducted during the summer and fall of 1979 with two plots, one under a mature stand of alfalfa and brome, and the other under barley. The design of the two plots is described in Figures 22 and 23. The grain plot was seeded with Galt barley on June 11 using a push-type, single-row plot seeder following a broadcast application of ammonium nitrate (34-0-0) and triple superphosphate (0-45-0) to the appropriate subplots and subsequent rototilling. The barley plot was weeded by hand over the summer. The alfalfa-brome plot received a broadcast application of triple superphosphate (0-45-0) onto the soil surface of the appropriate subplots on June 12. Fertilizer was broadcast to allow nondiscriminatory sampling of both plots, in spite of the reduced efficiency of this method application.

Soil samples of both plots on the Malmo soil were obtained at two depths (0-10 and 10-20 cm); each sample was a composite of six cores (2 cm in diameter) collected from each subplot with an Oakfield tube-type sampler. The composite samples were brought into the laboratory, sieved (less than 2 mm), mixed, and then randomly subsampled to provide two analytical replicates per subplot (each 1.4 g moist weight) for determination of phosphatase activity. The moisture content of the soil samples was determined at the same time as the phos-



Location: Ellerslie site (NE-24-51-25 W4)

Soil description: Malmo SiCL

Eluviated Black Chernozemic soil

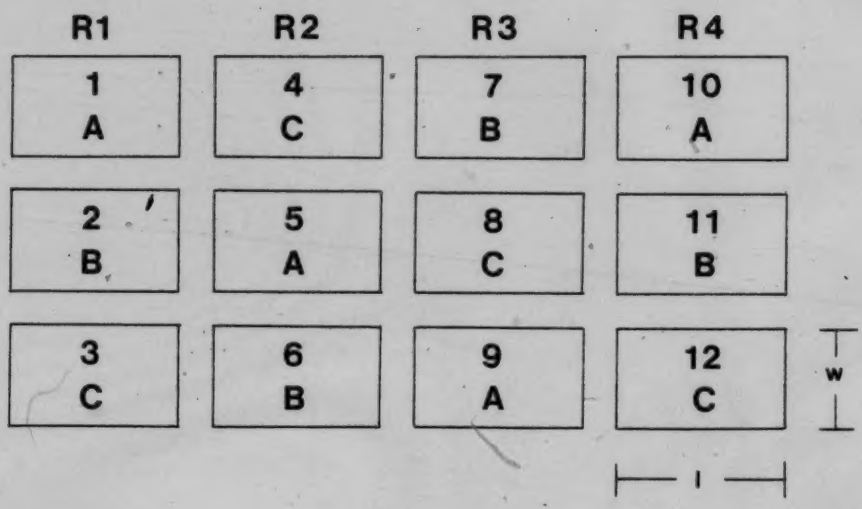
Crop: alfalfa and brome

Dimensions: subplots - 3 m x 5 m (w x l), with walkways 1 m wide

Treatments: A. control

B. triple superphosphate (0-45-0) at 40 kg P ha^{-1}

Figure 22. Design of plot under alfalfa and brome on the Malmo soil.



Location: Ellerslie site (NE-24-51-25 W4)

Soil description: Malmo SiCL

Eluviated Black Chernozemic soil

Crop: Galt barley seeded at approximately 160 kg ha^{-1} with 23 cm row spacings

Dimensions: subplots - 3 m x 5 m (w x l), with walkways 1 m wide

Treatments: A. control

B. ammonium nitrate (34-0-0) at 80 kg N ha^{-1}

C. N as above plus triple superphosphate (0-45-0) at 40 kg P ha^{-1}

Figure 23. Design of plot under barley on the Malmo soil.

phatase measurements. Alkaline (pH 8.0) phosphatase activity was determined by the method of Tabatabai and Bremner (1969) as described in chapter 2, in most cases within 48 hours of sample collection.

Plant samples were collected from both the alfalfa-brome and barley plots. The alfalfa-brome plot was sampled for above-ground plant material on July 31. Plant material was removed on each subplot from two separate, randomly selected areas (each 0.84 m^2). The plant material was cut with a hand sickle at 7-8 cm above the ground. The material was bagged, dried at 65 C for 48 hours, and weighed. The remaining plant material on the plot was cut with a mower on August 2, and removed. The barley plot was sampled on September 17, 18, 19, and 20. The above-ground plant material of the middle 3 m lengths of the middle 3 rows of each subplot (equivalent to an area of 2.07 m^2) was removed. Weights of plant material and grain were determined after drying.

4.2.3 Breton site

The samples from the Breton Plots (described by McCoy, 1973) were obtained from sieved (less than 2 mm), air-dry bulk soil samples of the Ap horizon which were collected in the fall of 1979 by Craig Miller and Graeme Spiers from the unlimed western half of the plots. Acid (pH 6.0) phosphatase activity was determined using the modified method described in chapter 3, with three analytical replicates per plot.

2.4 Statistical analyses

Analysis of variance and Duncan's multiple range test (where applicable) were computed for the phosphatase activity measurements to test for statistically significant effects and interactions. The phosphatase activity measurements (data to accompany Figures 24, 25, 26, and 27) are presented on pages 203-6.

4.3 Results

4.3.1 Ellerslie site

On the Malmo soil the alfalfa-brome and barley plots provided measurements of seasonal variation in alkaline phosphatase activity as affected by crop growth (Figures 24 and 25). There were several components of variation in the phosphatase activity measurements: replicates (representing spatial variation within the plot), time of sampling, fertilizer treatment, sampling depth, and analysis (representing variation within the composite sample). For both plots, the significant effects (at both confidence levels) were replicates, time, and depth (Tables 16 and 17). Furthermore, for the barley plot the interaction between time and depth was significant. The decrease in phosphatase activity with depth agrees with results reported by Speir (1977), Juma and Tabatabai (1978), and Speir and Ross (1978). The significant differences in enzyme activity among replicates indicate significant spatial variation in the field, even over

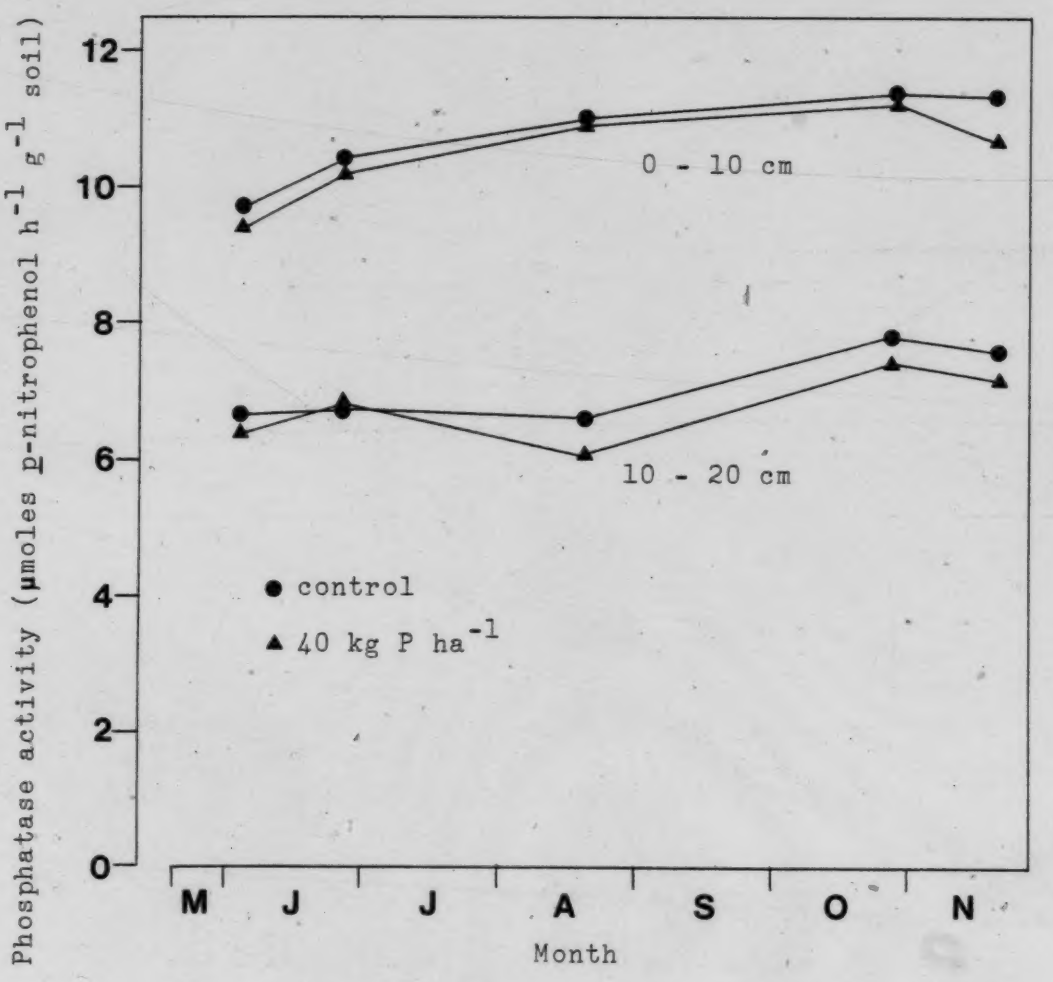


Figure 24. Seasonal variation in soil phosphatase activity at two depths under a stand of alfalfa and brome on a Malmo soil near Ellerslie, Alberta.

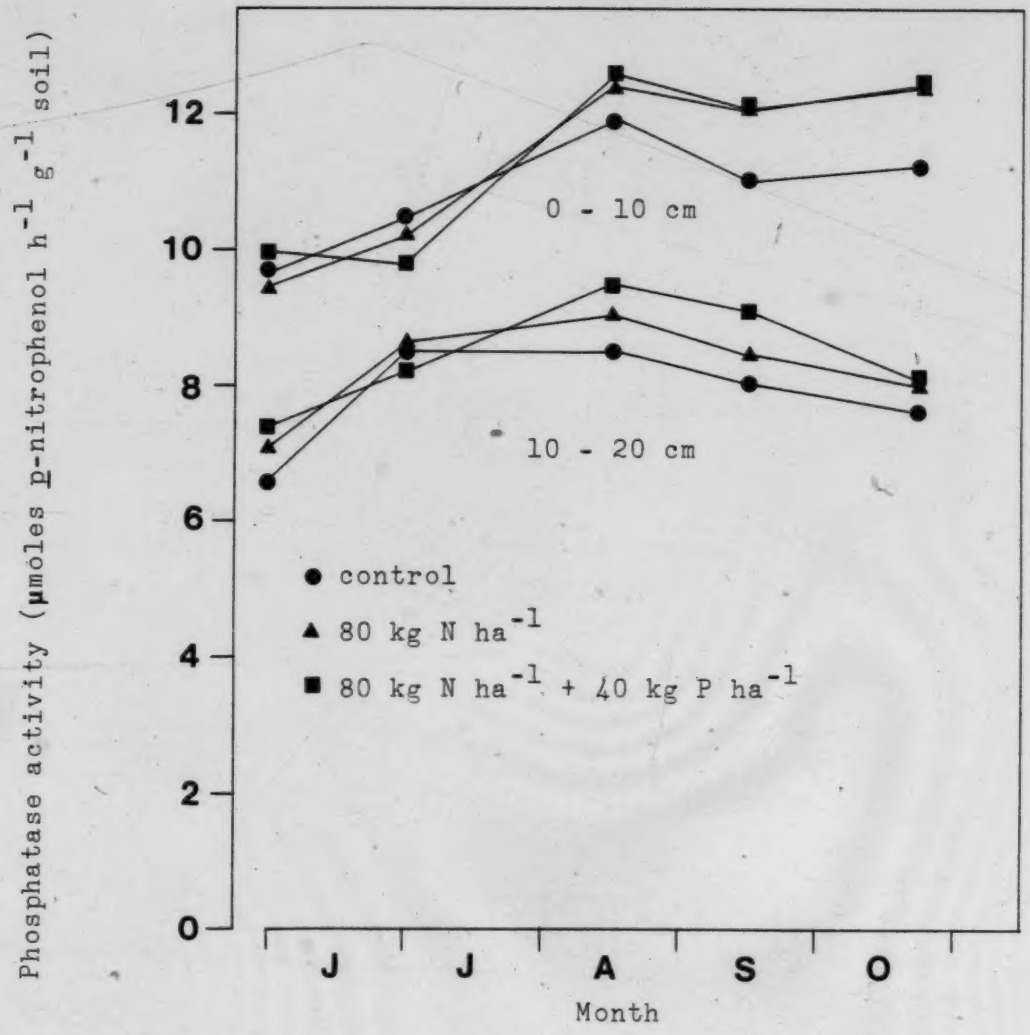


Figure 25. Seasonal variation in soil phosphatase activity at two depths under barley on a Malmo soil near Ellerslie, Alberta.

Table 16. Analysis of variance of phosphatase activity measurements of soil samples from the alfalfa-brome plot.

Source	DF	Sum of squares	Variance	F-value	Significance
Replicates	3	33.8	11.3	18.9	**
Time (T)	4	42.1	10.5	17.7	**
Error 1	12	7.15	0.596		
Subtotal 1	19	83.0			
Fertilizer (F)	1	3.42	3.42	0.45	N.S.
T x F	4	1.02	0.256	0.03	N.S.
Error 2	15	115	7.67		
Subtotal 2	39	119			
Depth (D)	1	524	524	261	**
Analysis (A)	1	3.31	3.31	1.64	N.S.
T x D	4	9.13	2.28	1.13	N.S.
T x A	4	0.435	0.109	0.05	N.S.
F x D	1	0.025	0.025	0.01	N.S.
F x A	1	0.380	0.380	0.19	N.S.
D x A	1	0.004	0.004	0.002	N.S.
T x F x D	4	1.05	0.262	0.13	N.S.
T x F x A	4	0.976	0.244	0.12	N.S.
T x D x A	4	1.14	0.286	0.14	N.S.
F x D x A	1	0.400	0.400	0.20	N.S.
T x F x D x A	4	0.542	0.136	0.07	N.S.
Error 3	90	181	2.01		
Total	159	723			

* significant at $P \leq 0.05$

** significant at $P \leq 0.01$

N.S. not significant

Table 17. Analysis of variance of phosphatase activity measurements of soil samples from the barley plot.

Source	DF	Sum of squares	Variance	F-value	Significance
Replicates	3	80.4	26.8	26.3	**
Time (T)	4	156	39.0	38.2	**
Error 1	12	12.2	1.02		
Subtotal 1	19	249			
Fertilizer (F)	2	15.5	7.77	0.82	N.S.
T x F	8	16.4	2.04	0.22	N.S.
Error 2	30	283	9.45		
Subtotal 2	59	315			
Depth (D)	1	543	543	53.8	**
Analysis (A)	1	0.570	0.570	0.20	N.S.
T x D	4	40.4	10.1	3.47	**
T x A	4	15.8	3.94	1.36	N.S.
F x D	2	0.127	0.064	0.02	N.S.
F x A	2	0.258	0.129	0.04	N.S.
D x A	1	0.015	0.015	0.01	N.S.
T x F x D	8	4.69	0.587	0.20	N.S.
T x F x A	8	3.22	0.402	0.14	N.S.
T x D x A	4	7.81	1.95	0.67	N.S.
F x D x A	2	0.138	0.069	0.24	N.S.
T x F x D x A	8	1.51	0.188	0.06	N.S.
Error 3	135	393	2.91		
Total	239	1.01×10^3			

* significant at $P \leq 0.05$

** significant at $P \leq 0.01$

N.S. not significant

relatively short distances. Harrison (1979) and Harrison and Pearce (1979) also observed appreciable spatial variation in phosphatase activity of soil. For both plots on the Malmo soil, there was a significant increase in phosphatase activity over the growing season coincident with plant growth, and extending into the fall. A similar trend was observed by Khaziyev (1967) and Gavrilova *et al.* (1973) in agricultural soils, and Harrison and Pearce (1979) for the averaged values of 48 woodland soils when expressed on a weight basis (g^{-1} soil). The seasonal variation in phosphatase activity in the present study represents variation in levels of active enzyme and hence potential activity, but not variation in actual phosphatase activity *in situ*. The significant interaction between time and depth for the barley plot indicates that there was more seasonal variation in phosphatase activity at the 0-10 cm depth than at the 10-20 cm depth.

Analyses of variance indicate that there was not a significant effect of fertilizer treatment on soil phosphatase activity of either plot over a single season. A single application of P at 40 kg ha^{-1} did not reduce enzyme activity relative to the control for the alfalfa-brome plot. The difference in weights of plant material produced by the two fertilizer treatments on the forage plot was not significant (Table 18); stand variation, however, may have masked the effect of fertilizer P on plant growth. Although application of N at 80 kg ha^{-1}

Table 18. Weight of plant material collected from the alfalfa-brome plot on a Malmo soil near Ellerslie, Alberta.

Treatment	Dry weight of plant material (g)*
A. control	352 ± 75
B. 40 kg P ha^{-1}	417 ± 141

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 8 values representing 2 randomly selected areas (each 0.84 m^2) per subplot.

with or without P at 40 kg ha^{-1} increased plant production relative to the control on the barley plot (Table 19), it did not produce significant differences in soil phosphatase activity among treatments. For both plots, however, the form of analysis of variance tested the effect of fertilizer with a relatively low degree of precision. A comparison of the two plots indicates that phosphatase activity under barley reached higher levels, and was more variable over five sampling dates, than that under the stand of alfalfa and brome.

4.3.2 Breton site

In soil samples of the Breton Plots there was lower acid phosphatase activity in the wheat-fallow rotation than in the five-year rotation of wheat, oats, barley, hay, and hay (Figures 26 and 27). Khan (1970) obtained similar results 10 years earlier. For the wheat-fallow rotation, the two halves (treated as replicates) yielded significantly different phosphatase activity values at the 0.05 confidence level, but not at the 0.01 level (Table 20). There appeared to be appreciable differences among treatments, but with only two replicates these were not statistically significant. For the five-year rotation there were significant differences in enzyme activity among series, fertilizer treatments, and analyses at both confidence levels (Table 21). Duncan's multiple range test indicated that phosphatase activity under the

Table 19. Weight of plant material collected from the barley plot on a Malmo soil near Ellerslie, Alberta.

Treatment	Dry weight of plant material (g)*	Dry weight of grain (g)*
A. control	428 ± 64	193 ± 33
B. 80 kg N ha^{-1}	769 ± 82	351 ± 48
C. 80 kg N ha^{-1} + 40 kg P ha^{-1}	864 ± 273	381 ± 123

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 4 values, each representing a sampling area of 2.07 m^2 per subplot.

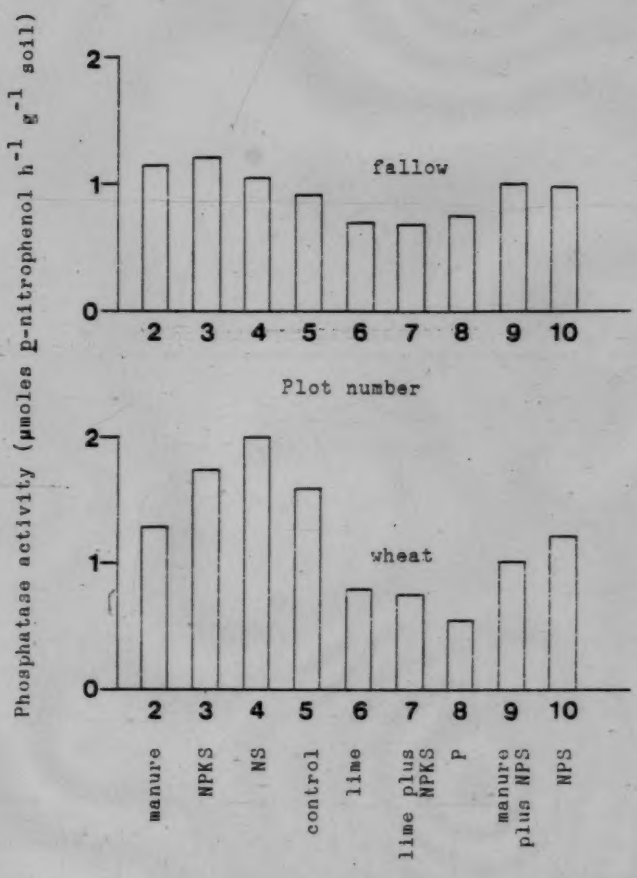


Figure 26. Acid phosphatase activity of the Breton L-SiL under a wheat-fallow rotation.

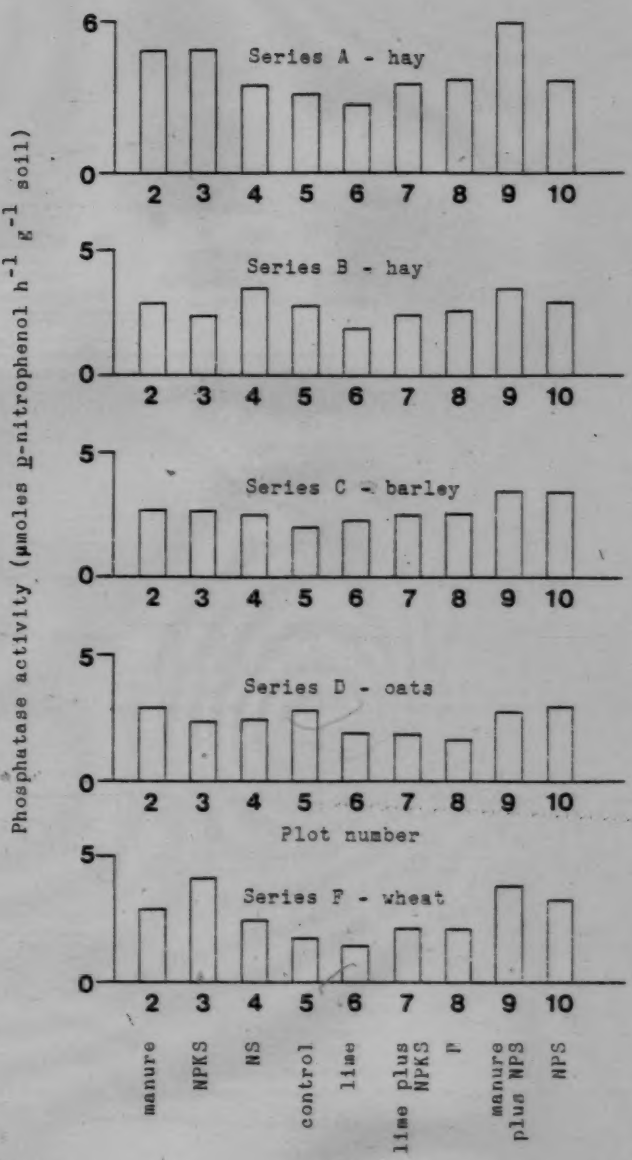


Figure 27. Acid phosphatase activity of the Breton L-SiL under a five-year rotation of wheat, oats, barley, hay, and hay.

Table 20. Analysis of variance of phosphatase activity measurements of soil samples from the wheat-fallow rotation of the Breton Plots.

Source	DF	Sum of squares	Variance	F-value	Significance
Replicates (Halves)	1	1.15	1.15	5.74	*
Treatments (T)	8	5.00	0.625	3.11	N.S.
Error 1	8	1.61	0.201		
Subtotal 1	17	7.76			
Analysis (A)	2	0.0249	0.0125	0.28	N.S.
T x A	16	0.413	0.0258	0.57	N.S.
Error 2	18	0.814	0.0452		
Total	53	9.01			

* significant at $P \leq 0.05$

N.S. not significant

Table 21. Analysis of variance of phosphatase activity measurements of soil samples from the five-year rotation (wheat, oats, barley, hay, hay) of the Breton Plots.

Source	DF	Sum of squares	Variance	F-value	Significance
Replicates (Series)	4	44.3	11.1	14.3	**
Treatments (T)	8	39.1	4.89	6.30	**
Error 1	32	24.8	0.776		
Subtotal 1	44	108			
Analysis (A)	2	1.27	0.633	6.49	**
T x A	16	1.70	0.106	1.09	N.S.
Error 2	72	7.03	0.098		
Total	134	118			

** significant at $P \leq 0.01$

N.S. not significant

second year of hay (series A) was significantly higher than that under the other crops, at both protection levels (Table 22). With the exception of plot 7 (lime plus NPKS), the fertilizer applications which would be expected to increase plant production (manure plus NPS, NPKS, manure, NPS, and NS) tended to produce the higher phosphatase activity values (Table 23). Plots 5, 6, 7, and 8 (control, lime, lime plus NPKS, and P respectively) produced the lower enzyme activity values; the effect of lime was pronounced, yielding the two lowest values. The significance of differences among analyses may be an artifact of the form of analysis of variance employed.

4.4 Discussion

The sampling and analytical procedures employed in the present study partly determined the specific component of soil phosphatase activity represented in the enzyme activity measurements. Field moist samples of Malmo soil were assayed for alkaline phosphatase activity immediately following collection, whereas air-dried Breton soil samples were assayed for acid phosphatase activity. Hence there was a larger microbial component in the Malmo samples (relative to the Breton samples); the acid phosphatase measurements for the Breton soil represent activity of stabilized, extracellular enzyme. These distinctions affect interpretation of the results.

Table 22. Duncan's multiple range test for significance of differences in acid phosphatase activity of soil samples among series of the five-year rotation (wheat, oats, barley, hay, hay) of the Breton Plots.

Series + crop	Acid phosphatase activity (μmoles p-nitrophenol h^{-1} g^{-1} soil)*	
A. hay	3.99	a
B. hay	2.74	b
C. barley	2.64	b
F. wheat	2.63	b
D. oats	2.36	b

* at both protection levels (0.05 and 0.01); values not followed by the same letter are significantly different from each other.

Table 23. Duncan's multiple range test for significance of differences in acid phosphatase activity of soil samples among treatments of the five-year rotation (wheat, oats, barley, hay, hay) of the Breton Plots.

Plot number + treatment	Acid phosphatase activity (μmoles p-nitrophenol h^{-1} g^{-1} soil)* at 0.05	at 0.01
9. Manure and NPS	3.87 a	3.87 a
3. NPKS	3.26 ab	3.26 ab
2. Manure	3.24 ab	3.24 ab
10. NPS	3.19 ab	3.19 ab
4. NS	2.86 bc	2.86 bc
8. P	2.53 bcd	2.53 bc
5. Control	2.47 cd	2.47 bc
7. Lime + NPKS	2.44 cd	2.44 bc
6. Lime	2.00 d	2.00 c

* values not followed by the same letter are significantly different from each other.

The results obtained in the present study confirm the first of the two hypotheses tested. Plant growth over the season increased levels of phosphatase in bulk samples of the Malmo soil under a stand of alfalfa and brome, and under barley. The significant interaction between time and depth under barley (indicating greater seasonal variation at the 0-10 cm depth) is further evidence for the influence of plant growth on enzyme activity, which is greater in the surface layer. The differences in acid phosphatase activity among series of the Breton Plots provide additional evidence of the effect of plant growth on enzyme levels in soil, and suggest differences in phosphatase production in the rhizosphere among crops.

The results of the present study corroborate those of Neal (1973), and support his conclusion that potential phosphatase activity in a soil can be altered by the growth of certain plants. This study did not, however, provide evidence for or against the hypothesis that higher phosphatase levels increase plant uptake of P. For both plots on the Malmo soil, phosphatase levels remained high into the fall, extending past the period of plant growth (particularly in the barley plot). This may represent microbial phosphatase activity associated with decomposition of plant residues. Similarly, seasonal variation in phosphatase activity in a Brown Chernozemic soil under native grassland showed highest activity (over

the growing season and fall) in November (Halm et al., 1972). Harrison and Pearce (1979) reported that phosphatase activity measurements for surface soils of 48 woodlands, when averaged and expressed on a weight or volume basis, indicated maximum levels of enzyme in December. They suggested that the winter peak in activity resulted from leaching of phosphatases from leaf-litter (shown to possess high phosphatase activity) which accumulated and decomposed on the soil surface. Harrison (1979) also reported high levels of phosphatase during the winter months in surface samples of 10 woodland soils.

The present study did not provide strong evidence in support of the second hypothesis that application of fertilizer inorganic P represses synthesis of phosphatase by plant roots and microorganisms. Nevertheless, the relatively low phosphatase activity values obtained for the P treatment of the Breton Plots (prior to 1965, approximately 5 kg P ha^{-1} yr^{-1}; from 1965 onwards, 6 kg P ha^{-1} yr^{-1}) may partly represent this phenomenon. The relatively high phosphatase levels of fertilizer treatments of the Breton Plots which included P with other nutrients (manure plus NPS, NPKS, manure, and NPS) and the apparent increases in phosphatase levels from fertilizer application to the barley plot on the Malmo soil may be explained by a sampling effect in which increased plant production due to fertilizer application increased the amount of rhizosphere soil included in the bulk samples.

Rhizosphere soil generally contains higher levels of phosphatase due to greater numbers of microorganisms present (Greaves and Webley, 1965), and because of plant production of phosphatase (Neal, 1973; Boero and Thien, 1979); hence increased amounts of rhizosphere soil in the bulk samples collected for analysis increased the average level of phosphatase in the samples. Such an indirect effect of fertilizers which increases phosphatase levels in the soil samples collected would be expected to mask repression of phosphatase synthesis by inorganic P. Spiers and McGill (1979) observed that P fertilization at 54 kg ha^{-1} yr^{-1} for 5 years tended to increase phosphatase activity of a Gray Luvisolic soil, an effect similar to that described above. In the Breton Plots, higher organic matter content resulting from increased plant production due to fertilizer application (Table 24) would also be expected to increase the level of phosphatase (Khan, 1970; Speir and Ross, 1978).

Results obtained for the plots on the Malmo soil indicate that changes in the level of phosphatase in bulk soil samples resulting from a single application of fertilizer (80 kg N ha^{-1}, 80 kg N ha^{-1} plus 40 kg P ha^{-1}, or 40 kg P ha^{-1}) were not reflected (at a significant level) in the seasonal pattern of activity. For the barley plot in particular, the differences in phosphatase activity between fertilizer treatments and the control apparent in Figure 25 were not statistically significant when

Table 24. Organic C values of soil samples of the Ap horizon from the Breton Plots (personal communication, Karen Cannon).

Plot number + treatment	% organic C*
2. Manure	1.78
3. NPKS	1.48
4. NS	1.46
5. Control	1.34
6. Lime	1.36
7. Lime + NPKS	1.48
8. P	1.35
9. Manure + NPS	2.05
10. NPS	1.70

* by Leco induction furnace

separated from the effect of time. With samples of the same soil, Spiers and McGill (1979) reported that P applied at 27 or 54 kg ha^{-1} yr^{-1} for 5 years (as opposed to a single application in the present study) reduced acid phosphatase activity by about 20%. Furthermore, with the plots on the Malmo soil, the broadcast method of application of triple superphosphate may have contributed to the absence of an effect of inorganic P on phosphatase activity. The broadcast application (followed by incorporation by rototilling on the barley plot) was employed to allow indiscriminate sampling of the plots for phosphatase activity analysis, and to maintain similarity in the method of application between the alfalfa-brome and barley plot. For forage production and no-till systems, Mays et al. (1980) concluded that there can be good uptake of surface-applied P. For the barley plot, however, as the season progressed the plants would have had to rely increasingly on P supplied from soil-fertilizer reaction products.

The acid phosphatase activity measurements for air-dried samples of the Breton Plots represent the average level of stabilized enzyme in the bulk samples. Hence significant differences between series and treatments would be expected to represent larger differences in enzyme levels in situ, which include an appreciable microbial and plant root component. Further, for the Malmo soil samples (assayed at field moisture content

immediately following collection), variation in the average phosphatase level of bulk samples suggests larger differences at the microsite. The choice of assay pH (8.0 for Malmo samples and 6.0 for Breton samples) was based on optimum values obtained from previous measurements on air-dried soil samples; use of an acid pH for assay of phosphatase activity of the Malmo samples may have detected larger changes in enzyme levels because of demonstrated plant root production of acid phosphatase.

The two limed treatments of the Breton Plots (plots 6 and 7) were associated with the two lowest phosphatase activity values. Prior to 1965 plot 7 received only lime and P. The pH values reported for the Breton Plots (Table 25) indicate higher values for the limed plots. Halstead (1964) reported that in incubated samples of seven acid soils addition of lime ($Ca(OH)_2$, $CaCO_3$, or $MgCO_3$) inhibited phosphatase activity determined by release of phenol or phosphate from disodium phenyl phosphate. Chloride and sulfate salts of Ca and Mg had no appreciable effect on phosphatase measurements, and activity rather than synthesis of enzyme was affected. The effect of lime on phosphatase activity of acid soils requires further investigation.

In the present study, for samples of the plots on the Malmo soil characterized by high phosphatase activity, depth was more important than sampling time, and both depth and sampling time were more important than fertil-

Table 25. Values of pH determined in 0.01 M $CaCl_2$ for soil samples of the Ap horizon from the Breton Plots (personal communication, Karen Cannon).

Plot number + treatment	Series A	Series B	Series C	Series D	Series E fallow	Series E wheat	Series F
2. Manure	5.7	5.2	5.3	5.5	5.8	5.6	5.8
3. NPKS	4.7	4.5	4.8	4.8	5.6	5.4	5.0
4. NS	4.9	4.8	4.7	5.1	5.6	5.6	5.4
5. Control	5.4	5.2	5.3	5.7	6.0	5.9	5.8
6. Lime	5.4	5.8	5.9	6.0	6.3	6.2	6.3
7. Lime + NPKS	5.8	5.8	5.8	5.9	6.2	6.2	5.9
8. P	5.3	5.6	5.6	5.8	6.1	5.9	5.7
9. Manure + NPS	5.4	5.2	5.5	5.6	5.8	5.8	5.6
10. NPS	4.9	4.9	5.3	5.2	5.7	5.7	5.2

izer treatment, as sources of variation in phosphatase activity over a single season. For samples of the Breton Plots, representing 50 years of specific cropping systems and fertilizer applications, rotation, crop, and fertilizer treatment were significant sources of variation in phosphatase activity. Harrison (1979) concluded that depth was more important than season or site as a source of variation in phosphatase activity of soils of 10 woodlands.

4.5 Conclusions

The results of this study support the following conclusions:

(i) there is substantial spatial and temporal variation in phosphatase levels in soils, which must be taken into account in field studies.

(ii) plant growth over the growing season increases the level of phosphatase in the rhizosphere. The increase in levels of phosphatase over the season includes a microbial contribution of enzyme, particularly in the fall (after the period of plant growth) when plant residues are being decomposed.

(iii) the present study did not provide evidence in support of the hypothesis that application of fertilizer inorganic P reduces phosphatase levels in the field by repression of phosphatase synthesis by plant roots and microorganisms.

(iv) expression of control of soil phosphatase activity in the field by inorganic P supply may not be clearly reflected in the pattern of spatial and temporal variation of enzyme levels _in situ_. The effect of inorganic P in repression of phosphatase synthesis is a documented microsite event; its expression in the field is often masked by a sampling effect in which increased plant production due to fertilizer application increases the amount of rhizosphere soil included in the sample, and hence the average level of enzyme in the sample.

(v) results obtained in the present study are similar to those of Khan (1970), indicating little change in the relative levels of phosphatase in the Breton soil over the last 10 years.

(vi) liming of acid soils decreases their acid phosphatase activity.

Experimental Data

Table 1. Seasonal variation in alkaline phosphatase activity of the Malmo SiCL under alfalfa and brome.*

Date	Depth	Treatments	
		A. control	B. 40 kg P ha^{-1}
May 28	0 - 10 cm	9.7 ± 0.65	9.3 ± 1.1
	10 - 20 cm	6.6 ± 0.50	6.4 ± 1.7
June 26	0 - 10 cm	10.4 ± 0.61	10.2 ± 0.56
	10 - 20 cm	6.8 ± 0.74	6.9 ± 1.0
August 14	0 - 10 cm	10.9 ± 0.62	10.8 ± 0.99
	10 - 20 cm	6.6 ± 1.0	6.1 ± 1.5
October 21	0 - 10 cm	11.3 ± 0.89	11.2 ± 1.1
	10 - 20 cm	7.8 ± 1.1	7.4 ± 1.2
November 12	0 - 10 cm	11.3 ± 0.54	10.6 ± 1.2
	10 - 20 cm	7.6 ± 1.0	7.2 ± 1.1

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 8 determinations; units - µmoles p-nitrophenol h^{-1} g^{-1} soil.

Table 2. Seasonal variation in alkaline phosphatase activity of the Malmo SiCL under barley.*

Date	Depth	Treatments		
		A. control	B. 80 kg N ha^{-1}	C. 80 kg N ha^{-1} + 40 kg P ha^{-1}
June 7	0 - 10 cm	9.6 ± 0.57	9.3 ± 0.81	9.9 ± 1.1
	10 - 20 cm	6.5 ± 1.6	7.0 ± 1.0	7.3 ± 0.49
July 9	0 - 10 cm	10.4 ± 1.4	10.2 ± 0.89	9.7 ± 1.5
	10 - 20 cm	8.5 ± 1.3	8.6 ± 0.42	8.2 ± 0.49
August 21	0 - 10 cm	11.8 ± 0.53	12.4 ± 1.3	12.5 ± 1.2
	10 - 20 cm	8.5 ± 1.0	9.0 ± 1.2	9.4 ± 0.54
September 25	0 - 10 cm	10.9 ± 1.1	12.0 ± 1.0	12.1 ± 1.4
	10 - 20 cm	7.9 ± 0.59	8.4 ± 0.58	9.1 ± 0.75
October 29	0 - 10 cm	11.2 ± 1.5	12.4 ± 0.95	12.5 ± 1.6
	10 - 20 cm	7.6 ± 1.2	7.9 ± 1.0	8.0 ± 0.78

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 8 determinations; units - µmoles p-nitrophenol h^{-1} g^{-1} soil.

Table 3. Acid phosphatase activity of the Breton L-SiL under the wheat-fallow rotation of the Breton Plots.*

Plot number + treatment	Series E1 (fallow)	Series E2 (wheat)
2. Manure	1.15 ± 0.13	1.31 ± 0.11
3. NPKS	1.19 ± 0.15	1.75 ± 0.62
4. NS	1.06 ± 0.13	2.01 ± 0.02
5. Control	0.89 ± 0.07	1.59 ± 0.32
6. Lime	0.69 ± 0.09	0.79 ± 0.14
7. Lime + NPKS	0.68 ± 0.04	0.76 ± 0.02
8. P	0.76 ± 0.06	0.55 ± 0.10
9. Manure + NPS	1.01 ± 0.08	1.07 ± 0.11
10. NPS	0.98 ± 0.06	1.22 ± 0.09

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 3 determinations; units - μmoles p-nitrophenol h^{-1} g^{-1} soil.

Table 4. Acid phosphatase activity of the Breton L-SiL under the five-year rotation (wheat, oats, barley, hay, hay) of the Breton Plots.*

Plot number + treatment	Series A (hay)	Series B (hay)	Series C (barley)	Series D (oats)	Series F (wheat)
2. Manure	4.89 ± 0.35	2.89 ± 0.28	2.73 ± 0.08	2.86 ± 0.19	2.86 ± 0.09
3. NPKS	4.92 ± 0.73	2.36 ± 0.21	2.64 ± 0.13	2.31 ± 0.28	4.05 ± 0.40
4. NS	3.39 ± 0.42	3.53 ± 0.48	2.48 ± 0.12	2.42 ± 0.13	2.46 ± 0.39
5. Control	3.09 ± 0.18	2.78 ± 0.30	1.97 ± 0.14	2.75 ± 0.19	1.75 ± 0.42
6. Lime	2.74 ± 0.28	1.88 ± 0.06	2.19 ± 0.45	1.80 ± 0.13	1.41 ± 0.39
7. Lime + NPKS	3.59 ± 0.82	2.31 ± 0.23	2.44 ± 0.19	1.80 ± 0.23	2.06 ± 0.11
8. P	3.69 ± 0.45	2.62 ± 0.37	2.60 ± 0.39	1.67 ± 0.15	2.06 ± 0.30
9. Manure + NPS	5.95 ± 0.26	3.44 ± 0.34	3.36 ± 0.22	2.76 ± 0.35	3.84 ± 0.50
10. NPS	3.65 ± 0.18	2.87 ± 0.53	3.32 ± 0.29	2.91 ± 0.06	3.17 ± 0.31

* $\bar{X} \pm s$, where $\bar{X}$ is the mean of 3 determinations; units - μmoles p-nitrophenol h^{-1} g^{-1} soil.

CHAPTER 5

SUMMARY

Controls on soil phosphatase activity were investigated under laboratory and field conditions in three experimental studies. The study reported in chapter 2 characterized phosphatase activity of air-dry surface samples of two soils (a Black Chernozemic soil and a Gray Luvisolic soil) in terms of pH optima and kinetic constants. Experimental results support the following conclusions:

(i) the different activity-pH curves for the two soils indicate differences in the relative amounts of plant and microbial phosphatase present, and indicate a predominance of microbial (alkaline) phosphatase in the Black Chernozemic soil.

(ii) the data suggest a multiple enzyme-environment system, even in dried soils which are dominated by stabilized enzyme.

(iii) the similarity in K_m values for phosphatase activity of air-dry samples of the two soils supports the hypothesis that microenvironment influences the activity of accumulated phosphatase.

(iv) differences in K_m values for samples of the two soils and those of plant and microbial phospha-

tases in solution may result from the effect of microenvironment in reducing affinity of enzyme for substrate.

The study reported in chapter 3 investigated control of phosphatase activity by orthophosphate in surface samples of a Gray Luvisolic soil over two successive incubation periods, each following a pulse of nutrients into the system. Results indicate that:

(i) synthesis of acid phosphatase by the proliferating microbial population in incubated soil samples was more responsive to derepressing conditions than was synthesis of alkaline phosphatase.

(ii) studies of controls on phosphatase production in incubated soil samples are more complicated than chemostat studies of microorganisms grown in solution culture because a significant amount of the phosphatase produced in amended soil by microorganisms can be stabilized and remain active.

(iii) although extractable P measurements detected changes in the level of labile inorganic P associated with repression and derepression of microbial synthesis of phosphatase, they did not clearly establish the role of recently synthesized enzyme in mineralization.

(iv) in the field appreciable derepression of phosphatase synthesis by microorganisms occurs with the addition of a readily oxidizable substrate in soil

with a low level of labile inorganic P and little recently synthesized enzyme.

The study reported in chapter 4 examined temporal and spatial variation of phosphatase activity in bulk samples of soil from the field, and related the effect of crop, rotation, and fertilizer (especially inorganic P) on enzyme levels in the field to controls on phosphatase activity by orthophosphate which operate at the microsite. Results indicate that:

(i) there is substantial spatial and temporal variation in phosphatase levels in soils, which must be taken into account in field studies.

(ii) plant growth over the growing season increases the level of phosphatase in the rhizosphere. The increase in levels of phosphatase over the season includes a microbial contribution of enzyme, particularly in the fall (after the period of plant growth) when plant residues are being decomposed.

(iii) the present study did not provide evidence for the hypothesis that application of fertilizer inorganic P reduces phosphatase levels in the field by repression of phosphatase synthesis by plant roots and microorganisms.

(iv) expression of control of soil phosphatase activity in the field by inorganic P supply may not be clearly reflected in the pattern of spatial

and temporal variation of enzyme levels *in situ*. Repression of enzyme synthesis by inorganic P in the field may be masked by a sampling effect in which increased plant production due to fertilizer application increases the amount of rhizosphere soil included in the sample, and hence the average level of enzyme in the sample.

(v) liming of acid soils decreases their acid phosphatase activity.

The conclusions of these studies provide a basis for comment upon the importance of soil phosphatase activity, and the utility of phosphatase activity measurements in soil testing. Phosphatase activity is involved in the cycling of P in soil (especially in undisturbed situations), specifically in supplying P to decomposers via derepression of enzyme synthesis in absence of sufficient labile inorganic P. Moreover, phosphatase activity of soil is one of several factors which determine the size, and rate of recharge of the soil labile P pool. Phosphatase activity cannot be used indiscriminately as an index of soil fertility or available P status without consideration of the controls on enzyme synthesis and activity. It may, however, have an application as a biological test of available P status of soil, because it assesses the capacity of soil to supply P to a growing organism as opposed to the size of a chemical fraction defined by the extract solution. The dynamic com-

ponent of phosphatase activity in soil is associated with a proliferating microbial population (as opposed to stabilized enzyme); plant root enzyme may also be important, but is difficult to examine in situ isolated from microbial enzyme.

Derepression of phosphatase synthesis by the proliferating microbial population (reported in chapter 3) represents the microbial response to an inadequate supply of orthophosphate, and initiation of "biochemical mineralization" as defined by McGill and Cole (1981)— release of P from organic form through enzymatic hydrolysis external to the cell membrane. Although plant root phosphatases may facilitate direct utilization of organic P in soil, biochemical mineralization of organic P by soil microorganisms and subsequent release of biomass P to the labile pool may contribute indirectly to plant uptake of mobilized P. Results of the present study suggest that biochemical mineralization, conceptualized by McGill and Cole (1981) as a flow from soil organic matter separate from that of C, N, and S, involves enzyme associated with or recently synthesized by an actively growing microbial population; the event requires a supply of readily oxidizable substrate for microbial growth, and a limited supply (or rate of supply) of labile inorganic P.

Several problems require further investigation, and the results of experimental work reported herein suggest

hypotheses to be tested. Phosphatase activity of derepressed microbial populations may "pump" P from plant residues and soil organic P compounds into the labile P pool via microbial biomass, and in this regard must be examined as part of a microbial process (as opposed to a strictly biochemical process). Another problem which must be studied is whether phosphatase activity is involved in long-term benefits of residual fertilizer P through increases in the size of the labile P pool. The results of the present study also indicate that in further research phosphatase activity must be studied together with the specific organic P fractions affected by the enzyme activity. The organic P fraction which acts as the substrate pool for phosphatase in soil must be identified, and controls on turnover of the pool _in situ_ identified.

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